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Preparation & *in vitro* evaluation of ^{90}Y-DOTA-rituximab

Mythili Kameswaran, Usha Pandey, Ashutosh Dash, Grace Samuel & Meera Venkatesh*

Isotope Production & Applications Division, Bhabha Atomic Research Centre, Mumbai, India &
**Physical & Chemical Sciences, International Atomic Energy Agency (IAEA), Vienna, Austria*

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***Background & objectives*: Radioimmunotherapy is extensively being used for the treatment of non-Hodgkin's lymphoma (NHL). Use of rituximab, a chimeric anti-CD20 antibody directed against the CD20 antigen in combination with suitable beta emitters is expected to result in good treatment response by its cross-fire and bystander effects. The present work involves the conjugation of p-isothiocyanatobenzyl DOTA (p-SCN-Bn-DOTA) to rituximab, its radiolabelling with ^{90}Y and *in vitro* and *in vivo* evaluation to determine its potential as a radioimmunotherapeutic agent.**

***Methods*: Rituximab was conjugated with p-SCN-Bn-DOTA at 1:1 antibody: DOTA molar ratio. The number of DOTA molecules linked to one molecule of rituximab was determined by radioassay and spectroscopic assay. Radiolabelling of rituximab with ^{90}Y was carried out and its *in vitro* stability was evaluated. *In vitro* cell binding studies were carried out in Raji cells expressing CD20 antigen. Biodistribution studies were carried out in normal Swiss mice.**

***Results*: Using both radioassay and spectroscopic method, it was determined that about five molecules of DOTA were linked to rituximab. Radiolabelling of the rituximab conjugate with ^{90}Y and subsequent purification on PD-10 column gave a product with radiochemical purity (RCP) > 98 per cent which was retained at > 90 per cent up to 72 h when stored at 37°C. *In vitro* cell binding experiments of ^{90}Y-DOTA-rituximab with Raji cells exhibited specific binding of 20.7 ± 0.1 per cent with ^{90}Y-DOTA-rituximab which reduced to 15.5 ± 0.2 per cent when incubated with cold rituximab. The equilibrium constant K_d for ^{90}Y-DOTA-Rituximab was determined to be 3.38 nM. Radiolabelled antibody showed clearance via hepatobiliary and renal routes and activity in tibia was found to be quite low indicating *in vivo* stability of ^{90}Y-DOTA-rituximab.**

***Interpretation & conclusions*: p-SCN-Bn-DOTA was conjugated with rituximab and radiolabelling with ^{90}Y was carried out. *In vitro* studies carried out in Raji cells showed the specificity of the radiolabelled conjugate suggesting the potential uitability of the formulation as a radiopharmaceutical for therapy of NHL.**

Key words Non-Hodgkin's lymphoma - radioimmunotherapy - rituximab - ^{90}Y-DOTA-rituximab

Radioimmunotherapy using suitable therapeutic radionuclides linked to target-specific monoclonal antibodies has regained importance in nuclear medicine due to the ready availability of monoclonal antibodies directed against antigens expressed in various cancers[1,2]. Both immunotherapy and radioimmunotherapy based on anti-CD20 antibodies are being used for the treatment of patients with non-Hodgkin's lymphoma (NHL). Yttrium-90 (^{90}Y) labelled ibritumomab tiuxetan (Zevalin) and ^{131}I labelled tositumomab (Bexxar) which target the CD20 positive B cell tumours are approved radioimmunotherapy agents for treatment of NHL[3]. However, both Zevalin and Bexxar use anti-CD20 antibody of murine origin while rituximab is a chimeric antibody directed against the CD20 surface antigen on B lymphocytes and is approved for the treatment of CD20 positive NHL[4,5]. Rituximab labelled with β^- emitting radionuclides has been shown to result in an increased therapeutic efficacy[6,7]. Hence, there is an increased interest towards the use of rituximab for radioimmunotherapy of NHL.

The radioisotope ^{90}Y has numerous advantages as a therapeutic radionuclide in comparison to ^{131}I. The availability from ^{90}Sr/^{90}Y generator systems renders no carrier added (nca) radionuclide[8]. The absence of gamma emissions makes outpatient treatment a possibility in case of ^{90}Y[9] while the emission of gamma by ^{131}I may require isolation of the patient after therapy[10]. The ^{131}I labelled antibodies reportedly degrade after internalization into the tumour cells, resulting in the circulation of the degraded products in the bloodstream[10]. The 2.28 MeV β^- particles from ^{90}Y have an effective path length of about 5.3 mm in tissues. This path length gives rise to 'cross fire effect' leading to the irradiation of tumour cells not bound to the antibody, which is highly beneficial in patients with bulky or poorly vascularized tumours[11]. However, ^{177}Lu (^{177}Lutetium) is also a potential radioisotope for radioimmunotherapy applications, depending upon the availability[12,13].

In Zevalin, ^{90}Y is bound to the murine anti-CD20 monoclonal antibody via the bifunctional chelating agent tiuxetan [an isothiocyanate benzyl derivative of diethylenetriamine pentaacetic acid (DTPA)]. To minimize the non-target uptake of radiometals, bifunctional chelating agents which form highly stable complexes with metal ions are preferred for conjugating the radiometals to biomolecules such as peptides and monoclonal antibodies[14]. The macrocyclic amino carboxylic acid ligand DOTA (1, 4, 7, 10-tetraazacyclododecane-1,4,7,10- tetraacetic acid) and its derivatives are the preferred chelators for ^{90}Y and radiolanthanides as these are known to form complexes with very high thermodynamic and kinetic stability[14]. The minimal loss of chelated metal ion occurs *in vivo* in case of DOTA conjugated biomolecules[15]. In the present study, rituximab was conjugated with p-isothiocyanatobenzyl DOTA and radiolabelled with ^{90}Y. The radiolabelled conjugate was characterized and evaluated for its affinity to CD20 antigens by carrying out *in vitro* cell binding studies in Raji cells expressing CD20 antigen.

Material & Methods

Rituximab (MabThera®-10 mg/ml) was purchased from Roche Inc., Basel, Switzerland. Para isothiocyanatobenzyl DOTA (p-SCN-Bn-DOTA) was purchased from M/s. Macrocyclics (Dallas, TX, USA). Arsenazo III, Copper (II) chloride, Roswell Park Memorial Institute 1640 medium (RPMI) 1640, 4-(2 hydroxyethyl)-1-piperazineethane sulphonic acid (HEPES) and sodium bicarbonate were procured from Sigma, USA. Foetal bovine serum (FBS) for use as a growth supplement in cell culture was from GIBCO, USA. Raji and U937 cells were procured from National Centre for Cell Science (NCCS), Pune, India, and maintained in the laboratory.

PD-10 columns were purchased from M/s. GE Healthcare, USA. AMICON Ultracentrifugal filter devices (MWCO 10,000Da) were from Millipore, India. Radioactivity measurements were carried out on a well type NaI (Tl) detector (ECIL, India). Size exclusion HPLC (SE-HPLC) analyses were performed on a system (M/s. JASCO, Japan) equipped with a TSK gel column (G3000 SWXL; 30 cm×7.8 mm; 5 μm) along with SWXL Guard column from TOSOH Biosciences, USA) and coupled to a UV/visible detector and a radioactivity detector (Raytest, Germany). Isocratic elution was carried out with 0.05 M phosphate buffer containing 0.05 per cent sodium azide (*p*H 6.8) at 0.6 ml/min. Chromatograms were analysed using GINA STAR software (Raytest GmBH, Germany). UV absorbance measurements were carried out on a JASCO spectrophotometer (M/s. JASCO, Japan). Radioactivity measurements during the

biodistribution studies were performed on an integral line flat-bed NaI (Tl) Scintillation Detector (Harshaw, USA).

Production of ^{90}Y: Yttrium-90 for the study was obtained from the 4 GBq ^{90}Sr/^{90}Y electrochemical generator developed in-house. The detailed procedure for the electrochemical separation of ^{90}Y from ^{90}Sr has been reported earlier[16]. The levels of ^{90}Sr were determined by the extraction paper chromatographic technique reported earlier[17].

Conjugation of rituximab with p-isothiocyanatobenzyl DOTA (p-SCN-Bn-DOTA): The conjugation of rituximab with p-SCN-Bn-DOTA was carried out at 1:10 molar ratio of antibody to p-SCN-Bn-DOTA ligand. A two ml aliquot of rituximab (10 mg/ml) was concentrated to one ml using an AMICON Ultra-centrifugal filtration device (MWCO 10,000 Da) by centrifuging at 15,000 *g* for 30 min. The *p*H of the solution was adjusted to 9.0 with 0.2M sodium bicarbonate buffer. Appropriate amount of p-SCN-Bn-DOTA was added and the reaction mixture was incubated at room temperature (25°C) for 2 h followed by overnight incubation at 4°C. An aliquot of the mixture was kept aside for the determination of number of DOTA molecules bound to rituximab by radioassay[18]. The reaction mixture was centrifuged in AMICON Ultracentrifugal filter devices (MWCO 10,000Da) to remove unreacted p-SCN-Bn-DOTA. Buffer exchange into 0.05M ammonium acetate buffer (*p*H 5.5) as well as the complete removal of the free p-SCN-Bn-DOTA was established by repeated washings with 0.05M ammonium acetate buffer (*p*H 5.5). Protein concentration in the DOTA-rituximab conjugate was determined by Lowry's method[19]. Purified DOTA-rituximab conjugate in 0.05 M ammonium acetate (*p*H 5.5) was stored at 4°C till further use for labelling.

Determination of number of DOTA per antibody molecule: The average number of chelator (p-SCN-Bn-DOTA) molecules bound to rituximab was determined by two methods *viz*. radioassay using cold $^{89}YCl_3.6H_2O$ spiked with trace of ^{90}Y acetate[18] and by spectroscopic assay using Cu (II)-Arsenazo (III) complex[20].

(i) Radioassay - In order to determine the number of DOTA molecules bound per antibody, an aliquot of the DOTA-rituximab conjugation reaction mixture was taken. To this, 37 MBq of $^{90}YCl_3$ was added along with cold $^{89}YCl_3$. The reaction was carried out at 37°C for 2 h and the reaction mixture was purified by size exclusion chromatography using PD-10 column wherein elution was carried out using 0.05 M phosphate buffer (*p*H 7.4). One ml fractions were collected and the radioactivity associated with each fraction measured. The number of chelates attached per rituximab molecule was calculated from the ratio of activity associated with rituximab to the total radioactivity.

(ii) Spectroscopic assay using Cu (II)-Arsenazo (III) assay - The number of DOTA molecules bound to rituximab was also determined using the Cu (II)-Arsenazo (III) assay as reported elsewhere[20]. This method measures the change in absorbance of a solution containing Cu (II)-Arsenazo complex due to the transchelation of Cu (II) with the DOTA of the DOTA-rituximab conjugate. A stock solution consisting of 25 µM of Cu (II) and 50 µM of Arsenazo (III) in 0.15 M ammonium acetate, *p*H 7.0 was prepared. Serial dilutions of this reagent were made, the absorbance at 652 nm was measured and molar absorption coefficient (ε) calculated.

To determine the absorbance of the purified DOTA-rituximab conjugates, 100 µl of the one ml Cu reagent was replaced with 100 µl of appropriately diluted DOTA-rituximab conjugate and the absorbance was measured at 280 nm and 652 nm at regular intervals of five min up to 30 min until the readings stabilized.

Determination of integrity of the DOTA-rituximab conjugate: To determine whether any major changes had occurred in the antibody due to conjugation with p-SCN-Bn-DOTA, SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) of rituximab and DOTA-rituximab under non-reducing conditions at varying dilutions was carried out in a 12.5 per cent homogenous gel using Laemmli's method[21]. The loaded gels were run at a constant current of 75-100 mA for 2 h. The gels were stained with Coomassie Blue R250. The mobilities of rituximab and DOTA-rituximab conjugate were compared with that of standard molecular weight standards.

Radiolabelling of DOTA-rituximab conjugate with ^{90}Y: DOTA-rituximab conjugate (1 mg) was taken in 200 µl of 0.5 ammonium acetate buffer (*p*H 5.5) to which approximately 148-185 MBq (4-5 mCi) of ^{90}Y chloride

was added. The reaction was carried out at *p*H 5.5 for 2 h at 37°C. Characterization of the ^{90}Y-DOTA-rituximab as well as the radiolabelling yield were determined by size exclusion (SE)-HPLC on a TSK G3000 SWXL Gel Column using 0.05 M phosphate buffer, *p*H 6.8 as the mobile phase at a flow rate of 0.6 ml/min. Purification of the ^{90}Y-DOTA-rituximab reaction mixture was carried out on a PD-10 column with 0.05 M phosphate buffer (*p*H 7.4) for elution. The *in vitro* stability of the radioconjugates was determined at 48 and 72 h when stored at 37°C by HPLC.

Biological evaluation of ^{90}Y-DOTA-rituximab:

(i) In vitro cell binding studies - Raji cells (Burkitt's lymphoma) which express CD20 antigen on their surface[22] were used for carrying out the *in vitro* binding studies of ^{90}Y-DOTA-rituximab conjugate. Cells were grown to confluence in RPMI medium containing 10 per cent foetal bovine serum. After harvesting, $2x10^6$ cells (*i.e.* 2×10^7 cells/ml) were incubated with ^{90}Y-DOTA-rituximab (0.7nM) for 2 h at 37°C. After incubation, the cells were washed twice with 1 ml of 0.05 M phosphate buffer (*p*H 7.4) and centrifuged at 6750 *g* for 20 min at room temperature. The supernatant was aspirated and the radioactivity associated with the pellet was measured. To confirm the extent of non-specific binding, blank studies were carried out by incubation of same number of cells and ^{90}Y-DOTA-rituximab with an additional 100nM of cold rituximab under identical experimental conditions. In addition, binding studies with non-specific cells *viz* U937 that do not express CD20 antigen on its surface, were also carried out.

The quality of ^{90}Y-DOTA-rituximab was measured using 10^4 to 10^8 Raji cells/ml and the immunoreactive fraction "r" determined by the method of Lindmo *et al*[23].

(ii) Equilibrium binding studies[24] - Specific binding was measured at six different concentrations of ^{90}Y-DOTA-rituximab ranging from 0.17 to 33.3 nM to determine the equilibrium dissociation constant (K_d). Raji cells (2×10^6 cells) in 0.4 ml RPMI medium were incubated with ^{90}Y-DOTA-rituximab (0.17-33.3 nM in triplicates) for 2 h on a shaker at 37°C. Non-specific binding was measured by having a similar set of reaction tubes in which 25 µg (165 nM) of cold rituximab was added in addition to ^{90}Y-DOTA-rituximab for all the six concentrations. At the end of the incubation period, the cells were washed twice with 0.05M phosphate buffer containing 1 per cent bovine serum albumin, centrifuged and the supernatant separated from the cell pellet. Both the cell pellet and supernatant were measured for radioactivity. Specific binding was determined by subtracting the non-specific binding counts from the total bound counts at each concentration of the radioimmunoconjugate. A plot of the concentration of radioimmunoconjugate versus the per cent specific binding was constructed. The data were analyzed by Scatchard plot using Graphpad Prism 5 software (GraphPad Software, Inc., California, USA).

Pharmacokinetic studies: All the animal experiments were performed after obtaining approval from the institutional animal ethics committee. Biodistribution and pharmacokinetic behaviour of ^{90}Y-DOTA-rituximab conjugate was determined at 3, 24 and 48 h post injection (p.i.) in normal Swiss mice. In brief, Swiss mice weighing 20-25 g were administered with approximately 740 kBq of ^{90}Y-DOTA-rituximab (~15 nM of antibody) per animal in 0.1 ml via lateral tail vein. The animals were sacrificed at 3, 24 and 48 h (n=4 per time point), blood collected, organs dissected and weighed. Concomitant radioactivity was measured in a gamma counter and percentage of dose administered per gram (% ID/g) was determined.

Results

Conjugation of p-SCN-Bn-DOTA with rituximab: The number of DOTA molecules bound to the antibody was determined to be five, as per radioassay. This was also confirmed by spectroscopy assay which showed that six molecules of DOTA were bound to one molecule of antibody.

Integrity of the DOTA-rituximab conjugate: SDS-PAGE was carried out to determine the integrity of the DOTA-rituximab conjugate. On destaining the gel, it was observed that both rituximab and the DOTA-rituximab conjugate, under non-reducing conditions, showed comparable distinct bands at 150-160 kDa (Fig.1). This experiment indicated that no macroscopic changes occurred to the antibody on conjugation with 5-6 molecules of DOTA.

Purification and characterization of ^{90}Y-DOTA-rituximab conjugate: The ^{90}Y-DOTA-rituximab reaction mixture was purified by passing through a PD-10 column and eluted with 0.05 M phosphate

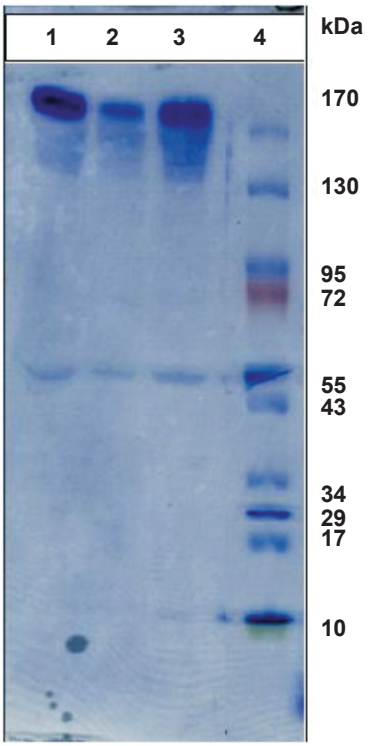

Fig. 1. SDS-PAGE pattern of rituximab and DOTA-rituximab conjugate under non-reducing conditions. Lane 1 - Rituximab Ab (50 µg), Lane 2 - Rituximab Ab (25 µg). Lane 3 - Rituximab-DOTA conjugate (50 µg), Lane 4 - Mol. wt. standards.

buffer (*p*H 7.4). The radiolabelled antibody conjugate was eluted in the fourth and fifth fractions which was comparable to the PD-10 elution pattern of the cold antibody conjugate that was independently determined by measuring the UV absorption at 280 nm. The purified ^{90}Y-DOTA-rituximab was characterized using SE-HPLC as shown in Fig. 2A. The ^{90}Y-DOTA-rituximab had a retention time of 15 min. HPLC analysis of ^{90}Y chloride performed under the same conditions showed a retention time of 22 min (Fig. 2B). The radiolabelled antibody could be obtained with >99 per cent radiochemical purity. The stability of the ^{90}Y-DOTA-rituximab conjugate was studied up to 72 h at 37°C and it was observed that the radiolabelled conjugate retained approximately 90 per cent radiochemical purity even after 72 h of storage at 37°C (Fig. 3).

In vitro cell binding studies: *In vitro* cell binding studies carried out in Raji cells showed a specific binding of 20.7 ± 0.1 per cent with ^{90}Y-DOTA-rituximab (0.7 nM) which reduced to 15.5 ± 0.2 per cent (25% inhibition) when incubated with 100 nM of cold rituximab indicating the specificity of ^{90}Y-DOTA-rituximab for CD20 antigen. Non-specific U937 cells showed only background counts.

The immunoreactive fraction "r" was determined by plotting a double inverse plot of total over specific binding as a function of the inverse cell concentration which was found to be 0.75 (*i.e.* 75%).

In the equilibrium binding experiments, it was observed that the uptake of ^{90}Y-DOTA-rituximab in Raji cells was concentration dependant. The specific binding of ^{90}Y-DOTA-rituximab with CD20 antigen was obtained after subtraction of non-specific uptake value from the cold tubes having excess of cold rituximab. Using the data for specific uptake, Scatchard plot was constructed using the Graph Pad Prism 5 program (Fig. 4). The K_d values as derived from the Scatchard plot (K_d= -1/slope) was 3.38 nM indicating high binding affinity of ^{90}Y-DOTA-rituximab to CD20 antigen.

Pharmacokinetic studies: The results of the biodistribution studies of ^{90}Y-DOTA-rituximab carried out in normal Swiss mice are shown in Fig. 5. The activity in blood and liver was initially very high which decreased with time. Even at 48 h p.i., there was significant activity resident in the spleen which is as expected with radiolabelled antibodies. The radiolabelled antibody showed both hepatobiliary clearance and renal clearance. Activity associated with the tibia was found to be less indicating *in vivo* stability of ^{90}Y-DOTA-rituximab.

Discussion

The availability of humanized monoclonal antibodies and a wide choice of therapeutic radionuclides in conjunction with improved strategies to target the radiolabelled agents to specific tumours have led to significant advances in radioimmunotherapy. Amongst the multitude of radioimmunotherapy agents pursued, by far the best results have been obtained in haematopoietic neoplasms especially NHL for which radiolabelled antibodies for therapy (Bexxar and Zevalin) are commercially available. With the availability of rituximab which can be radiolabelled with suitable therapeutic radioisotopes, it is expected to have promising results in the therapy of NHL. The potential use of ^{177}Lu-rituximab in the therapy of NHL has been reported[25,26]. Use of ^{90}Y for labelling rituximab was motivated by the fact that ^{90}Y ibritumomab tiuxetan

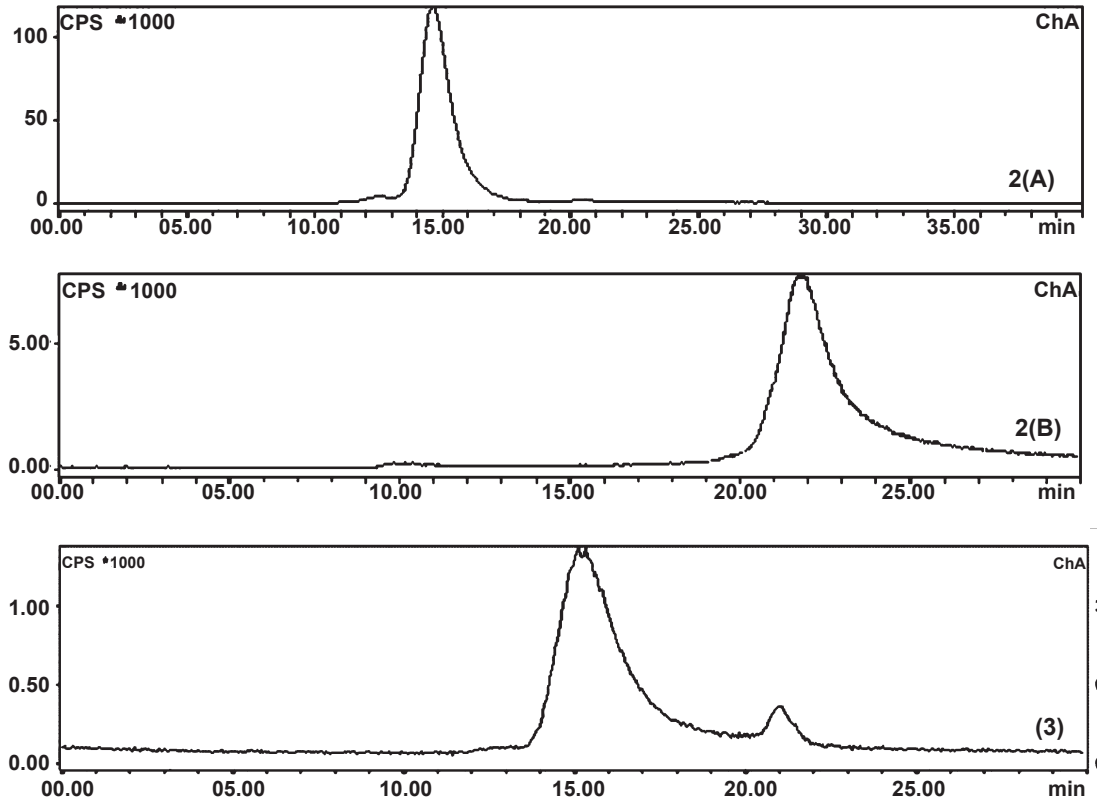

Fig. 2 (A). Size exclusion (SE)-HPLC pattern of pure ^{90}Y-DOTA-rituximab conjugate. **2(B).** SE-HPLC pattern of ^{90}Y chloride. **Fig. 3.** HPLC pattern of ^{90}Y-DOTA-rituximab conjugate (stored for 72 h at 37°C).

(Zevalin), a ^{90}Y labelled murine anti-CD20 antibody, is already an approved radiopharmaceutical for treatment of NHL. Yttrium-90 has no gamma emissions and hence patients who receive ^{90}Y therapy can be treated as outpatients. Radioimmunotherapy with Zevalin has been shown to be well tolerated and has clinically significant higher overall response rate (ORR) and complete response (CR) as compared to treatment with rituximab alone[27,28].

Conjugation of ^{90}Y with anti-CD20 antibody using suitable derivatives of the acylic bifunctional chelating agent DTPA has been reported[29-31]. However, the macrocyclic chelating agent DOTA has shown excellent kinetic inertness to metal ion release when complexed with ^{90}Y and other lanthanides[32]. The conjugation of rituximab was carried out with p-isothiocyanato benzyl DOTA as previous studies using ^{90}Y labelled DOTA conjugated antibodies have shown the superiority of DOTA as a bifunctional chelating agent in comparison to DTPA[15,33]. Increase in the number of DOTA molecules linked to the antibody adversely affects its

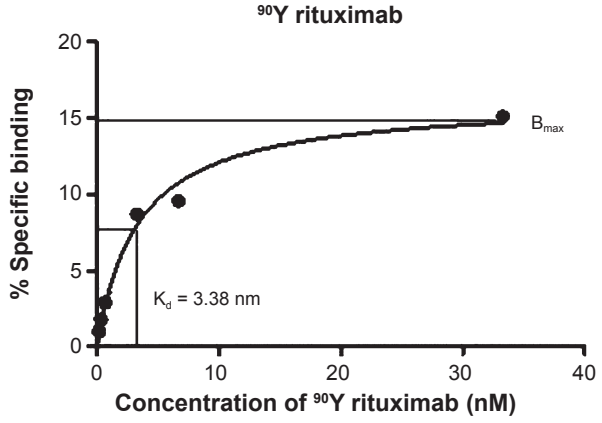

Fig. 4. Saturation binding of ^{90}Y-DOTA-rituximab conjugate (K_d).

pharmacokinetics resulting in increased liver uptake apart from decreasing the immunoreactivity of the antibody conjugate[25]. However, the immunoreactivity of the antibody is not significantly affected when conjugated with, upto five chelates per antibody molecule[33]. Hence, our experiments were carried out

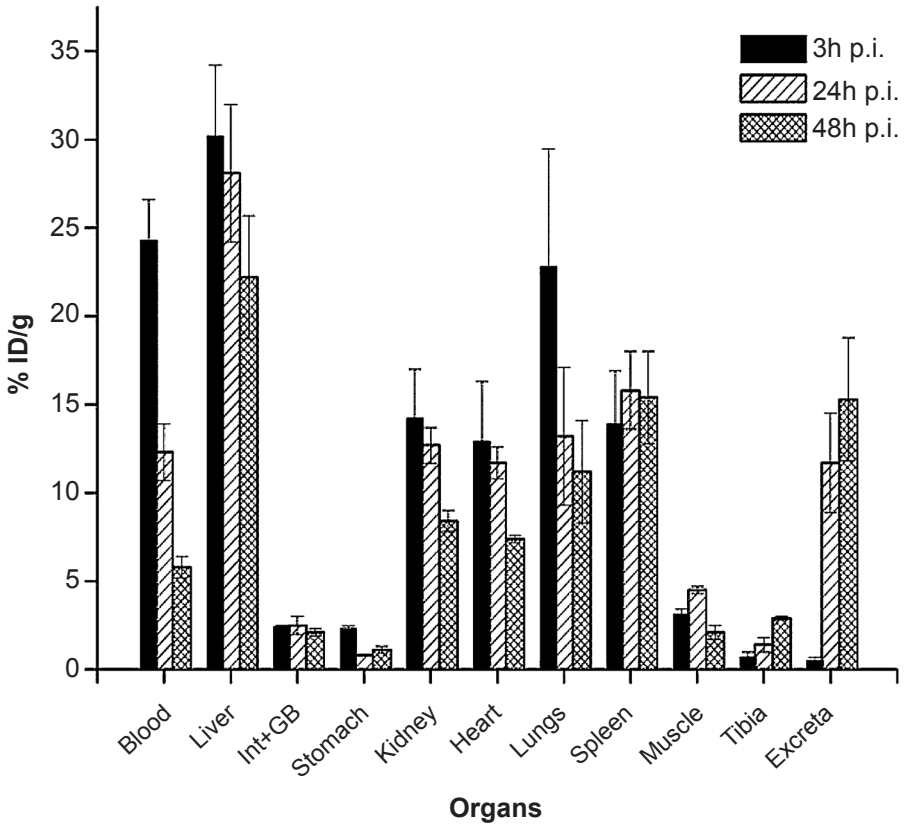

Fig. 5. Biodistribution pattern of ^{90}Y-DOTA-rituximab in normal Swiss mice. %ID/g, % injected dose/g.

using the DOTA-rituximab conjugate having five DOTA molecules per antibody molecule.

In vitro cell binding studies carried out in Raji cells showed high specificity of ^{90}Y-DOTA-rituximab for CD20 antigen. In the equilibrium binding experiments the K_d value of 3.38 nM obtained with the ^{90}Y-DOTA-rituximab was better than the values reported for Zevalin[34]. *In vitro* cell binding and equilibrium binding assays were carried out at 37°C as earlier reports stated that the equilibrium constant did not change significantly between 2 and 40°C[35]. It has also been reported that for antibodies under consideration for *in vivo* use, measurements should ideally be carried out at 37°C[36]. Pharmacokinetic studies carried out in normal mice confirmed the *in vivo* stability of the product, as indicated by the low uptake in tibia. However, detailed bioevaluation studies in tumour bearing animals would further confirm the specificity of the product.

In conclusion, p-isothiocyanatobenzyl DOTA was successfully conjugated to rituximab and the antibody conjugate was radiolabelled with ^{90}Y. The purified ^{90}Y-DOTA-rituximab conjugate with a radiochemical purity of >99 per cent exhibited excellent stability when stored at 37°C up to 72 h. Bioevaluation studies showed the specificity of the radiolabelled conjugate for CD20 antigen. The results indicate the potential of ^{90}Y-DOTA-rituximab for further evaluation as a radioimmunoconjugate to be used for therapy of NHL.

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***Conflicts of Interest*:** None.

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Reprint requests: Dr Mythili Kameswaran, Isotope Production & Applications Division, Bhabha Atomic Research Centre, Mumbai 400 085, Maharashtra, India
e-mail: kmythili@barc.gov.in