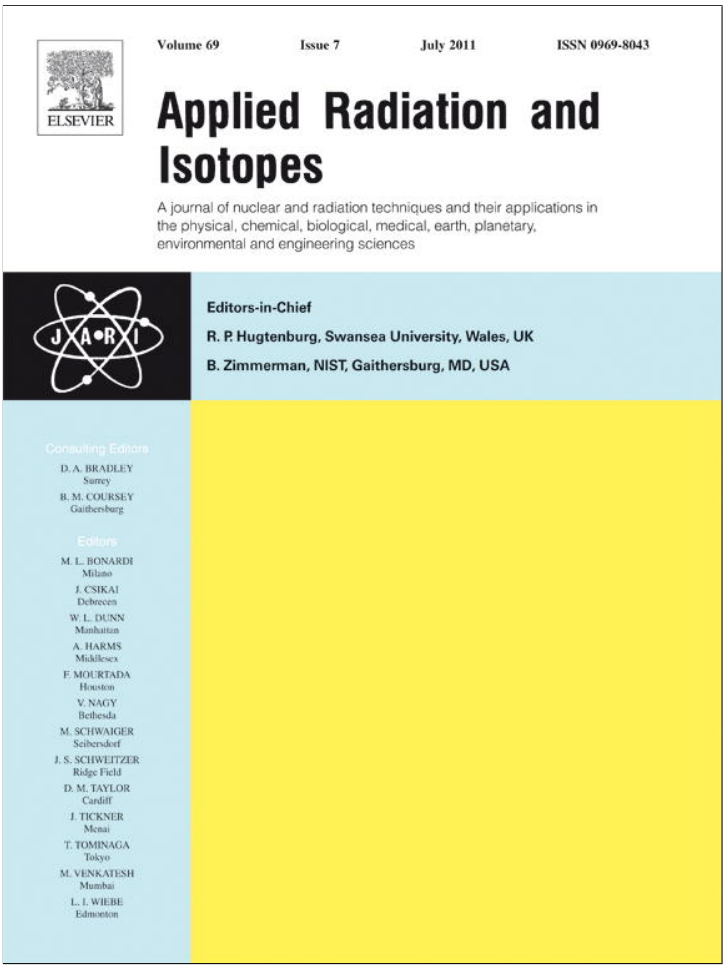

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[^{177}Lu]DOTA-anti-CD20: Labeling and pre-clinical studies

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ABSTRACT

Anti-CD20 (Rituximab®), a specific chimeric monoclonal antibody used in CD20-positive Non-Hodgkin's Lymphoma, was conjugated to a bifunctional quelate (DOTA) and radiolabeled with ^{177}Lu through a simple method. [^{177}Lu]-DOTA-anti-CD20 was obtained with a radiochemical purity higher than 97%, and showed good chemical and biological stability, maintaining its biospecificity to CD20 antigens. Monte Carlo simulation showed high doses deposited on a spheroid tumor mass model. This method seems to be an appropriate alternative for the production of [^{177}Lu]-DOTA-anti-CD20 as therapeutic radiopharmaceutical.

1. Introduction

Anti-CD20 (Rituximab®), a specific chimeric monoclonal antibody directed against CD20 surface antigen on B lymphocytes, is used in the treatment of CD20-positive Non-Hodgkin's Lymphomas (NHL) (Coiffier et al., 1998; Johnson and Glennie, 2003). Its association with beta emitter radionuclides enhances anti-CD20 therapeutic effectiveness (Davis et al., 2004; Kaminski et al., 1996; Witzig et al., 2002a, 2002b). Two therapeutic radiopharmaceuticals for radioimmunotherapy, anti-CD20 labeled with ^{131}I (Bexxar®) or with ^{90}Y (Zevalin®), have already been approved by FDA for the treatment of indolent, refractory or relapsed NHL. Ibritumomab (Zevalin®) is now accepted as first line treatment for follicular lymphoma (Juweid, 2002; Vose et al., 2000; Witzig et al., 2002a, 2002b). Lutetium-177 (^{177}Lu, Eβ mean 166 keV, Eγ 113 keV 6.5%, 208 keV 11%, *T*1/2 6.7 d) is a gamma and beta emitter radionuclide. Its gamma emission is used to obtain in vivo images of biodistribution and dosimetric studies, while beta emission produces the desired therapeutic effect. ^{177}Lu has several advantages for radionuclide therapy: low tissue penetration, scarce damage to normal surrounding tissues, low gamma energy radiation, low abundance and adequate lifetime (Milenic and Brechbiel, 2004).

Previous reports (Forrer et al., 2009) described the radiolabeling of anti-CD20 with ^{177}Lu by conjugation with DOTA through isotiocyanate-benzyl-DOTA showing good results. In the present report we study an alternative and simple method based on Lewis et al.'s (1994) report, to radiolabel anti-CD20 monoclonal antibody (Rituximab®) with ^{177}Lu through conjugation to 1,4,7,10-tetraaza cyclododecane-N, N, N′, N″′-tetraacetic acid (DOTA). In vitro and in vivo studies were carried out in order to demonstrate complex stability and behavior. Additionally, a dosimetric study was performed by Monte Carlo simulation software in order to survey the effects of this therapeutic radiopharmaceutical within the tumor mass as well as in the surrounding tissues.

2. Materials and methods

2.1. Preparation of DOTA-anti-CD20 conjugate

DOTA was activated by incubation with N-hydroxysulfosuccinimide (sulfo-NHS) and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) for 45 min at 4 °C (Lewis et al., 1994). The mixture was incubated with anti-CD20 at pH 8.5 for 18 h at 4 °C, and then purified by size exclusion chromatography.

2.2. Radiolabeling

For the labeling, 55.5–185.0 MBq (1.5–5.0 mCi) of [^{177}Lu]$LuCl_3$ (MURR, Missouri) and 100 μl of gentisic acid (10 mg/ml) were added to 0.5 mg of DOTA-anti-CD20; the resulting solution was incubated for 30 min at 37 °C. [^{177}Lu]-DOTA-anti-CD20 finally was purified by size exclusion chromatography (PD-10 column Sephadex G-25, Pharmacia) with ammonium acetate 0.25 M

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buffer at pH 7 as eluent, obtaining fractions of 0.5 ml. DOTA-anti-CD20 radiolabeling efficiency was evaluated by thin-layer and size exclusion chromatography.

Radiochemical purity controls were performed by three different chromatography systems; one using ITLC-SG (Pall Corporation) embedded in 5% BSA as support and ethanol:NH_4OH:H_2O (2:1:5) as mobile phase ([^{177}Lu]DOTA-anti-CD20=1), another using ITLC-SG as support and sodium acetate solution 14% as mobile phase ([^{177}Lu]DOTA-anti-CD20=0) and by size exclusion chromatography.

2.3. Stability studies

DOTA-anti-CD20 storage stability was studied at two different temperature conditions, 4 and −20 °C. After different storage periods (1, 3, 6 months), both were labeled at the same conditions. In order to evaluate this stability, radiolabeling yield was used.

[^{177}Lu]DOTA-anti-CD20 in vitro stability studies were performed by incubation in human serum and NaCl 0.9% at 37 °C, and in reaction buffer at room temperature for 72 h.

All control studies were performed after radiolabeling for different periods by thin layer chromatography using the supports and mobile phases described above. Another control system used was size exclusion chromatography with an elution volume of 3 ml for [^{177}Lu]DOTA-anti-CD20 and 6 ml for ^{177}Lu.

2.4. Immunoaffinity studies

Immunoaffinity studies (binding studies) were performed by incubation of the radiolabeled complex with membrane extract of human B cells rich in CD20 antigens. These cells were controlled by flow cytometry in order to ensure the presence of CD20 antigens. After 24 h of incubation, samples were centrifugated for 50 min at 3500 rpm, and the obtained pellets were measured in a scintillation counter (Compac 120 Picker, USA). The binding studies were performed in order to determine the maximum binding capacity (MBC) using a fixed amount of radiolabeled conjugate, and increasing concentrations of membrane extract, and the inhibition of binding to these membranes using a fixed amount of radiolabeled conjugate and a fixed concentration of membranes (6 mg/ml) with increasing concentrations of unlabeled anti-CD20 solution. Competition studies (inhibition 50% – IC50) were also performed.

2.5. In vivo animal studies

Two different biodistribution studies were performed in triplicate, using six weeks-old male CD-1 mice. In the first case, 22.6–40.7 MBq (0.6–1.1 mCi) of [^{177}Lu]DOTA-anti-CD20 were administered intravenously through the tail vein. Mice were euthanized at 4, 16 and 24 h post injection, organs of interest were removed, weighed and radioactivity measured in a solid scintillation counter (ORTEC).

The procedure mentioned above was also performed by administering increasing doses of unlabeled anti-CD20 (125, 250, 500 and 1000 mg/m^2) 3 h before the injection of the radiolabeled anti-CD20. Mice were euthanized at 16 h post injection.

All animal studies were conducted in compliance with national institutional animal care rules.

2.6. Monte Carlo simulation

Dosimetric evaluation was performed using the Monte Carlo PENELOPE package (Salvat et al., 2003), considering [^{177}Lu]DOTA-anti-CD20 uniformly distributed in a spheroid as tumor mass. The simulation main program was based on the sample PENCYL provided in the 2003 PENELOPE distribution, which was adapted in order to account for the spherical symmetry of the problem. An energy distribution for the primary electron beam was also introduced, according to the decay data provided in Nuclear Decay Data in the MIRD Format (IAEA).The simulations were carried out in a cluster facility with 3.0 MHz Pentium IV processors, each run with 10^7 primary electrons, which typically implied 3×10^5 s of CPU time. This ensures reasonable uncertainties for dose distributions, generally below 1% for all regions.

The routines included in the PENELOPE package generate random electron–photon showers in complex material structures consisting of any number of distinct homogeneous regions (bodies) of different compositions. The code allows the users to write their own simulation program, with arbitrary geometry and scoring. The main program, which is provided by the user, has to control the evolution of the simulated tracks and keep score of the relevant quantities. The simulation of electron and positron tracks is performed by means of a mixed algorithm (Berger, 1963). Individual hard elastic collisions, hard inelastic interactions and hard bremsstrahlung emission are simulated in a detailed way, by means of a random sampling from the corresponding restricted differential cross sections. This procedure ensures reliable simulation results, while saving CPU time.

The isotropic primary emission was simulated according to the capability of the PENCYL package for extended sources, in which the user can choose the emission direction of the primary electrons. No path length limitation has been imposed in the simulations performed, since interactions within any region (e.g. the tumor region) should be favoured. In addition, conservative values were chosen for elastic scattering parameters and cutoff energy losses for inelastic collisions and bremsstrahlung emission, according to the suggestions given in Salvat et al. (2003).

In order to generate dose distribution plots in the tumor volume and surroundings, the deposited energy was integrated in spherical shell volume of constant radius for this reason, the cumulative dose attributed to low energy particles may not be negligible, which was accounted by taking a low energy cutoff level of 10 keV. Two different approaches were faced: on the one hand, the radionuclide was taken as uniformly distributed along the tumor mass; on the other hand, the drug intake was assumed to occur only in the tumor spherical surface. Although as a first approximation these can be thought as equivalent situations, it is clear that in the first case, an important dose delivery will occur within the tumor mass, whereas emission only from the surface could remarkably affect the surrounding tissues. The aim of the simulation faced is to survey the real situation for the beta energies involved in this method.

3. Results and discussions

DOTA is a bifunctional chelating agent widely used in the synthesis of radioconjugates because it forms very stable complexes with many metals (Korde et al., 2007; Ogawa et al., 2009; Das et al., 2007). Compared with other bifunctional agents such as DTPA, DOTA provides more stable complex in vivo and in vitro (Lewis et al., 1994). Anti-CD20 monoclonal antibody was conjugated with this bifunctional ligand DOTA by a simple method and was purified by PD-10 column recovering more than 97%. The complex was stable in buffer reaction both at 4 and −20 °C, and it was stored up to six months. Therefore, this method produces a stable conjugate suitable to store DOTA-anti-CD20 in a simple way and to use it routinely.

Labeling yield of [^{177}Lu]DOTA-anti-CD20 ranged from 75% to 79% and radiochemical purity after purification was higher

than 97%. The highest specific activity reached about 300 MBq/mg (8.1 mCi/mg), which is suitable for clinical use (Forrer et al., 2009). Radiochemical purity controls using the systems described before, allowed to separate different radiochemical species present in the reaction with a fast and simple method. [^{177}Lu]DOTA-anti-CD20 was stable up to 72 h in reaction buffer at room temperature. These results are comparable with results of Forrer et al. (2009).

In vitro radioimmunoconjugate stability evaluation showed that [^{177}Lu]DOTA-anti-CD20 was stable for at least 72 h in both 0.9% NaCl and human serum. These results were adequate to proceed with the biological evaluation of radiolabeled anti-CD20.

Flow cytometry of the human B cells used for bioaffinity studies showed 10% expression of CD20 antigen.

Specific binding of [^{177}Lu]DOTA-anti-CD20 to membrane cell extract increased as function of membrane concentration (Fig. 1). Maximum binding capacity (MBC) was 35 ± 5%. Inhibition of binding to these membranes was 78.1 ± 2.1% when 2.2 pM of unlabeled anti-CD20 solution was added. The value obtained from IC50 was 8.98 ± 2.78 nM. These results show that [^{177}Lu]DOTA-anti-CD20 maintains its specific biological activity and high affinity to CD20 antigens. The methodology developed for biologic controls allows a fast, accurate and reproducible evaluation, and also, the use of membrane antigen extract makes it unnecessary to work with whole human cells whose manipulation is more difficult and introduces more variables in the test.

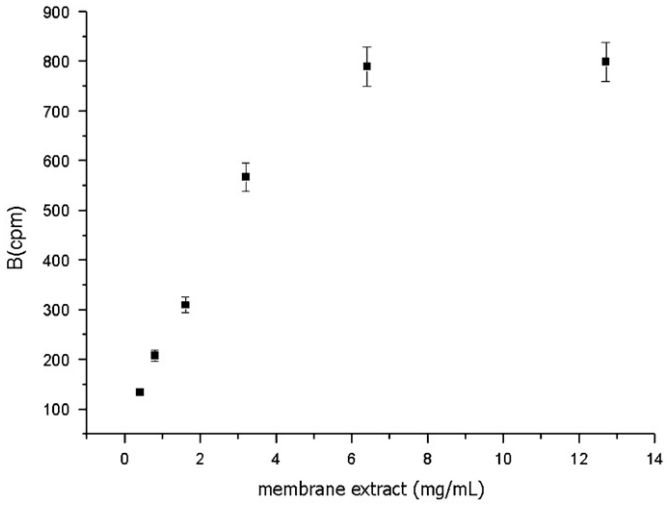

Fig. 1. [^{177}Lu]-DOTA-anti-CD20 bound versus membrane extract concentration.

Biodistribution studies showed the main routes of elimination were hepato-biliary and urinary. Liver uptake (17.8 ± 0.2%, 24.7 ± 0.6% and 41.0 ± 6.2% at 4, 16 and 24 h, respectively) and urinary bladder uptake (11.6 ± 0.5%, 5.1 ± 0.1% and 5.2 ± 2.5% at 4, 16 and 24 h, respectively) was observed in normal mice with no significant uptake in other organs. The great in vivo stability of the radioconjugate is indicated by the bone low uptake considering the high affinity of free ^{177}Lu to bone tissue (Breeman et al., 2003). (Fig. 2)

A correlation was sought between mouse body surface and unlabeled anti-CD20 dose often administered to patients by body surface for treatment according to accepted protocols for Zevalin® and Bexxar®. Studies with different doses of unlabeled anti-CD20 showed a decrease in liver uptake and an increase in urinary elimination, while blood activity remains practically constant. The saturation of the liver microsomal system by unlabeled anti-CD20, results in a decrease of liver uptake of ^{177}Lu-DOTA-anti-CD20. (Fig. 3)

^{177}Lu beta emission energy (mean: 166 keV) is lower than other radionuclides commonly used for this therapy (mean ^{131}I 191 keV, ^{90}Y 699 keV). Some reports show that low energies result in best tumor to non-tumor ratio dose than higher beta emission energies (Bernhardt et al., 2001a, 2001b). For this reason, ^{177}Lu would be more appropriate than ^{131}I and ^{90}Y for treatment. Although it is known that the distribution of antigens in a tumor is not homogeneous, Monte Carlo simulations were carried out within a simplified scheme of uniform antigen

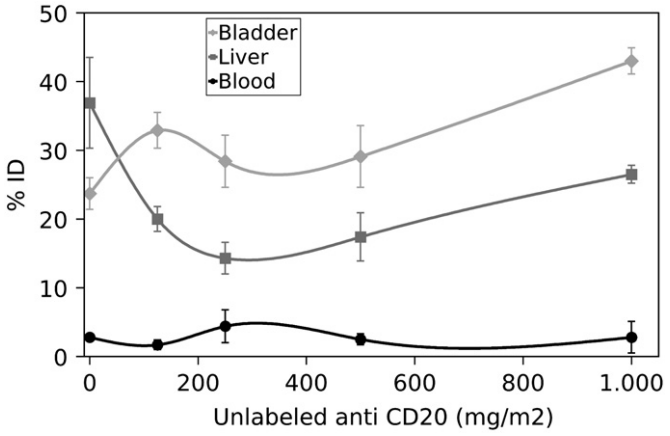

Fig. 3. Variation in the uptake of [^{177}Lu]-DOTA-anti-CD20 against the previous dose of unlabeled anti-CD20 administered. (Experimental data have been smoothly connected only for clear visualization).

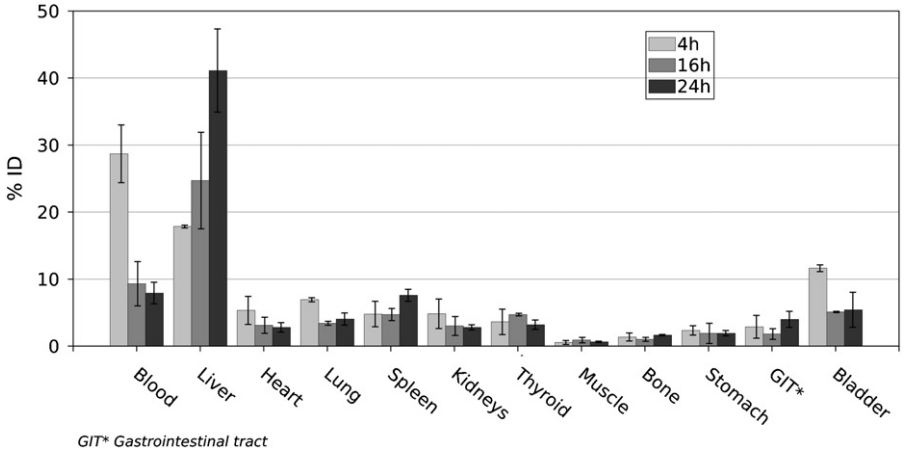

Fig. 2. Biodistribution of [^{177}Lu]-DOTA-anti-CD20 in normal mice at 4, 16 and 24h.

distribution for dose deposition estimates. A homogeneous spheroid tumor mass of 0.5 cm diameter was assumed, and two extreme cases were considered for [^{177}Lu]DOTA-anti-CD20 uptake; on one hand, uniform distribution throughout the tumor mass and on the other hand, uniform distribution only along the spheroid surface. In this study only beta particles are considered, since gamma emission does not contribute to tumor dose deposition. These calculations showed 80% of the dose was deposited within the tumor mass, whereas only 20% of the dose affected surrounding non target tissue (Fig. 4). For the tumoral model considered, ^{177}Lu was more suitable than ^{131}I or ^{90}Y because it produces less toxic radiation damage on normal tissues surrounding the tumor. As described above, a second approach was also faced, in which [^{177}Lu]DOTA-anti-CD20 was homogeneously distributed only in the tumor spherical surface; in this case, which would be the most unfavorable situation, 45% of the dose was still deposited in the targeted tissue, with a rather more important irradiation of healthy surrounding cells (Fig. 5).

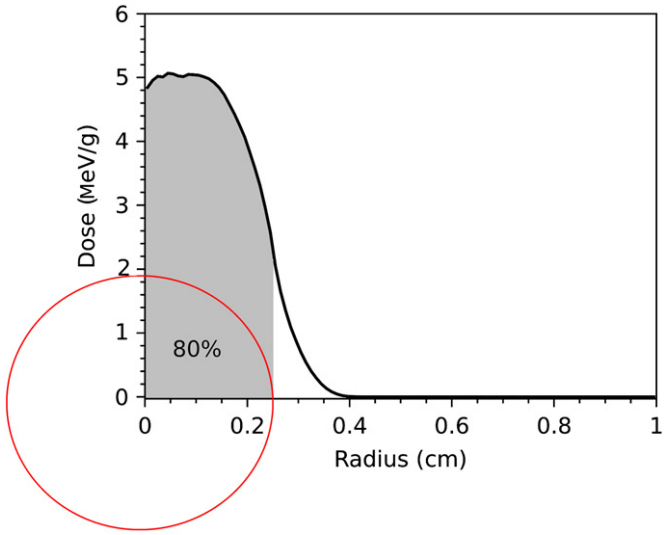

Fig. 4. Monte Carlo simulation results for dose deposited by [^{177}Lu]-DOTA-anti-CD20 distributed uniformly throughout a spheroid tumor mass model of 0.5 cm in diameter.

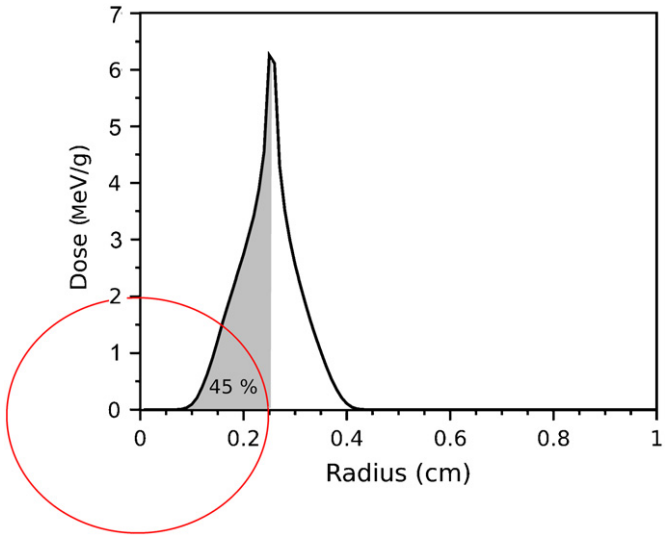

Fig. 5. Monte Carlo simulation results for dose deposited by [^{177}Lu]-DOTA-anti-CD20 distributed uniformly in the surface of a tumor spherical model of 0.5 cm in diameter.

4. Conclusions

Anti-CD20 was labeled by a fast and simple method, obtaining a biological and chemically stable radioimmunoconjugate. This is an alternative method to produce [^{177}Lu]DOTA-anti-CD20, with an appropriate radiochemical purity. This becomes a potential clinical application as therapeutic radiopharmaceutical for the treatment of NHL. Monte Carlo simulations showed that ^{177}Lu is more suitable for treatment of small and medium size tumor masses, as compared to other radionuclides commonly used for this type of treatment.

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