REPORT

2nd Research Coordination Meeting
on

"Development and preclinical evaluation of therapeutic radiopharmaceuticals based on ^{177}Lu- and ^{90}Y- labelled monoclonal antibodies and peptides:"

Stip, Republic of Macedonia
October 01-05, 2012

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Italy (Rome)
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Syria
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1. INTRODUCTION

The Coordinated Research Project (CRP) entitled 'Development and preclinical evaluations of therapeutic radiopharmaceuticals based on ^{177}Lu and ^{90}Y labelled monoclonal antibodies and peptides' has been organized by IAEA with the major purpose of promoting the introduction in Member States of clinical applications based on radioimmunotherapy (RIT) and peptide receptor-based radionuclide therapy (PRRNT) for some important cancers. Targeted radionuclide therapy with labeled monoclonal antibodies (mAbs) and peptides has emerged as a complementary modality for the treatment of cancer. Several mAbs have been introduced for immunotherapy of various cancers. These include Rituximab® (for B-cell lymphomas), Trastuzumomab® (for breast cancer), Alemtuzumab® (for chronic lymphocytic leukemia), Cetuximab® (colorectal, head & neck cancers), and Bevacizumab® (for colorectal cancers). Two radiolabeled mAbs, Zevalin® (labeled with ^{90}Y) and Bexxar® (labeled with ^{131}I), have been approved for radioimmunotherapy (RIT) of B-cell lymphomas. Both these agents have demonstrated significant antitumour response following a single treatment in patients with B-cell lymphomas resulting in more than 40-70% complete responses (CR) lasting 3-7 years. Similarly, radiolabelled somatostatin-analog peptides have been extensively investigated as both diagnostic and therapeutic agents for neuroendocrine tumours, and some ^{177}Lu and ^{90}Y radiolabelled derivatives are currently under extensive clinical evaluation.

While RIT and PRRNT have been demonstrated to provide effective alternative treatments against some cancers, in developing countries, they do not yet constitute a firmly established therapeutic modality due to the high cost and low availability of some relevant radionuclides for routine clinical studies. This CRP was designed to develop technologies for the production of therapeutic radiopharmaceuticals based on monoclonal antibodies and peptides labeled with ^{177}Lu and ^{90}Y with the aim of promoting the clinical application of RIT and PRRNT. Recently, ^{177}Lu has attracted much interest as therapeutic radionuclide due to its suitable physical characteristics. ^{177}Lu is emerging as a clear choice for therapy due to its easy production logistics, suitable physical characteristics, such as soft beta emission (E_{max} of 497, 384 and 172.KeV with 78.6, 9.1, and 12.2% abundance, respectively), and relatively longer half-life of 6.71 days). Unlike ^{90}Y, ^{177}Lu also has gamma emissions {113 KeV (6.4%) and 208 KeV (11%)}, appropriate for imaging studies to document targeting and biodistribution studies for organ dosimetry calculations. The recent past activities of the IAEA through coordinated research projects have significantly contributed to enhancing the availability of both ^{90}Y and ^{177}Lu radionuclides. Yttrium-90 can be prepared in no-carrier added form from a ^{90}Sr/^{90}Y generator investigated through the CRP on 'Development of generator technologies for therapeutic radionuclides' (2004-2007). A CRP on 'Development of therapeutic radiopharmaceuticals based on ^{188}Re and ^{90}Y for radionuclide therapy' (2008-2011) further evaluated the technology underlying ^{188}W/^{188}Re and ^{90}Sr/^{90}Y generators and validated the quality control procedures of ^{90}Y and ^{188}Re eluates obtained from these generators. An important result of the above CRPs was the technology transfer occurred among the participants' groups that sharply enhanced the availability of clinical grade ^{90}Y in several countries. The technology for the production of high specific activity ^{177}Lu suitable for radiolabeling peptides and antibodies was demonstrated thorough the CRP on 'Development of therapeutic radiopharmaceuticals based on ^{177}Lu for radionuclide therapy' (2006-2009). The availability of the radionuclides ^{90}Y and ^{177}Lu in several member states might strongly enhance the applicability of targeted radionuclide therapy as an alternative approach for cancer treatment.

As mentioned above, among the various MAbs evaluated thus far, anti-CD20 mAbs specific for B-cell lymphomas have shown significant antitumor response and improved progression free survival either given alone or given as radioimmunoconjugates. Since Rituximab® will be available soon as a generic product, this CRP has been designed to focus on the preparation of either ^{177}Lu or ^{90}Y-labeled Rituximab as a therapeutic radiopharmaceutical for the treatment of lymphomas. Specifically, the development of a sterile kit formulation for Rituximab labelling was envisaged as a critical achievement because it could make possible the easy and safe in-house preparation of the radiolabeled MAb for RIT studies in patients. Similarly, the CRP was also expected

to facilitate the development of other ^{177}Lu and ^{90}Y therapeutic agents based on the labelling of peptides specifically targeting receptors expressed onto the surface of cancerous cells.

Participants of the consultant's meeting on 'Therapeutic radiopharmaceuticals for the treatment of cancers', held in August 23-27, 2010 at IAEA headquarters, provided key inputs for the formulation of this CRP. The first research coordination meeting (RCM) was held in Vienna on May 9-13, 2011. This Report illustrates the results of the second RCM meeting, which was organized by the University of Stip, Republic of Macedonia on October 01-05, 2012.

2. REVIEW OF RESULTS ACHIEVED DURING THE FIRST PART OF THE CRP

2.1. Monoclonal antibodies: Rituximab

Rituximab (formula, $C_{6416}H_{9874}N_{1688}O_{1987}S_{44}$; molecular weight, 144 kD) is a chimeric anti-CD20 B-cell specific monoclonal antibody approved for the treatment of low-grade non-Hodgkin's lymphoma. Therefore, Rituximab was almost exclusively employed by participants'groups for the development of a therapeutic agent for RIT labelled with ^{177}Lu and ^{90}Y. In the first part of this CRP, major efforts have been addressed to achieve a satisfactory optimization of techniques for conjugation of different bifunctional chelating agents (BFCA) to the monoclonal antibody (Moab), labelling conditions and quality control methods of the resulting conjugates. In particular, the following procedures have been extensively investigated to assess the most appropriate experimental conditions allowing the highest yield and degree of reproducibility.

- Conjugation of the monoclonal antibody to a bifunctional chelating agent (BFCA).
- Purification of the BFCA conjugated antibody (BFCA-MoAb).
- Chemical characterization of the resulting BFCA-MoAb.
- Labelling of the BFCA-MoAb with ^{177}Lu and ^{90Y}Y.
- Determination of the radiochemical purity (RCP) of the resulting radiolabelled conjugate (M-BFCA-MoAb; M = ^{177}Lu and ^{90Y}Y).
- Determination of the in-vitro stability of M-BFCA-MoAb (M = ^{177}Lu and ^{90Y}Y).

The chemical structures of the various BFCAs employed for the study are illustrated in Fig. 1 below. The compounds **1–4** were commercially available. Martin Brechbiel's group at National Institute of Health (NIH) provided compound **5** to all groups. The two BFCAs **1** and **4** have been employed more extensively.

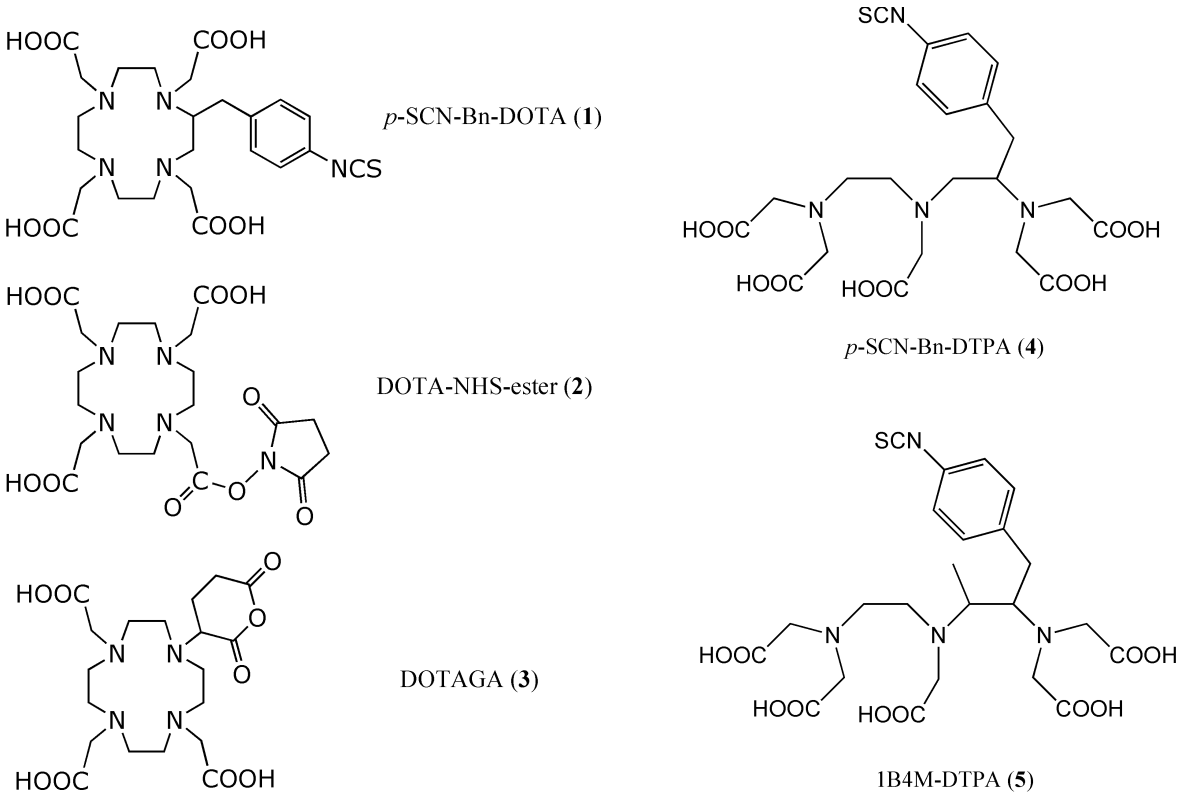

FIG. 1. The bifunctional chelating agents employed for conjugation to Rituximab.

Participants'groups presented results from their own studies obtained with the different chelating agents. These data have been extensively and carefully analyzed during the RCM with the purpose of identifying the most appropriate experimental protocol. The crucial issue was to clearly define a precise and detailed experimental procedure for all steps required to successfully conduct the preparation and quality control of the radiolabelled Rituximab. It was assumed that these standardized protocols have to be exactly followed by all research groups for conducting their subsequent studies on radiolabelled Rituximab during the second part of the CRP. The analysis of experimental data allowed drafting a number of protocols precisely describing each key step on the path for obtaining the high-yield labelling of the antibody including quality control methods. These procedures are reported here (see below) and all groups are strongly encouraged to be committed to following them closely in their future studies on the labelling of Rituximab.

The implementation of operational protocols for antibody conjugation and labelling has to be considered as a critical achievement of the CRP because it will allow for further progress towards the final objective of developing a kit formulation for routine clinical use. In fact, only the definition of exact protocols may permit to translate them into an easy-to-use kit-like procedure. Actually, the investigation to evaluate the most appropriate conditions for producing a lyophilized kit formulation for labelling Rituximab with ^{177}Lu and ^{90}Y has been already initiated by a few groups within the CRP. In particular, the group from Poland presented a preliminary kit formulation containing the conjugated MoAb, mannitol and sodium acetate in a freeze-dried form. Data reported by the same group showed that the kit formulation was sufficiently stable and afforded ^{177}Lu-Rituximab in high radiochemical yields. Similarly, the group from Macedonia investigated the impact of different lyophilisation conditions on the formation of the microcrystalline structure of the conjugated antibody. These studies will provide a preliminary basis for the successful development of a suitable kit formulation.

2.2. Peptides: bombesin

In the first part of the CRP, some groups were also pursuing the goal to investigate the preparation of a new peptide-based therapeutic radiopharmaceutical labelled with ^{177}Lu and ^{90}Y. During the first RCM, the peptide called substance-P was proposed as a suitable candidate for developing a therapeutic agent for the treatment of malignant gliomas. Although a number of research groups have successfully conducted labelling studies of substance-P with ^{177}Lu, it has been noticed that recent reports published on the scientific literature seriously questioned the potential utility of this peptide for targeting selectively brain tumours as receptors for substance-P are also expressed by a high number of healthy tissues. For this reason, participants' groups agreed to switch the interest for a new peptide-receptor ligand from substance-P to bombesin.

Peptidic bombesin derivatives are particularly attractive for the development of tumour-specifc therapeutic agents. Bombesin (BBN), a 14-aminoacid peptide analog of human gastrin releasing peptide (GRP) and of the related neuromedin B (NMB) peptide, was originally isolated from the skin of the frog Bombina bombina in 1970 [Anastasi, A., Erspamer, V., M. Bucci, M., Isolation and structure of bombesin and alytesin, two analogous active peptides from the skin of the European amphibians Bombina and Alytes. Experientia 27 (1971) 166-167] (Fig. 2).

FIG.2. Image of the frog Bombina bombina.

GRP and BBN share a common aminoacid sequence in the C-terminal region, which is necessary for exhibiting their biological activity. Four different bombesin receptor subtypes have been recognized in humans, namely the neuromedin B receptor (NMB-R), the gastrin-releasing peptide receptor (GRP-R), and the orphan bombesin subtype-3 receptor (BB_3). These receptors can be distinguished by their different affinities for the mammalian peptides NMB and GRP, whereas a native ligand for the BB_3 has not been identified yet [Varvarigou, A., et al., Gastrin-releasing peptide (GRP) analogues for cancer imaging. Cancer Biother Radiopharm, 19 (2004) 219-229]. The fourth BBN receptor (BB_4) was isolated from the frog but has not been found in mammals yet. The interest in using radiolabeled bombesin derivatives as agents for radionuclide therapy of tumours has increased because of the observation that GRP-R are over-expressed in a variety of human tumor cells [Chen, J., et al., Synthesis, stabilization and formulation of [^{177}Lu]Lu-AMBA, a systemic radiotherapeutic agent for gastrin releasing peptide receptor positive tumors. Appl Radiat Isot 66 (2008) 497-500]. GRP-R is not normally expressed by epithelial cells present in colon, lung, and prostate, but is expressed by non-neuroendocrine cells of the pancreas and breast and by most neuroendocrine cells of gastrointestinal tract, lung and prostate. However, GRP receptors are found in high density in a variety of primary and metastatic tumour tissues, such as breast, colon and prostate tumours. In the case of prostate cancer, it has been found a high density of GRP-R not only in the invasive prostatic carcinomas but also in the earliest phase of neoplastic transformation. GRP-R expression by prostate tumour cells seems to be closely related to the onset of the neoplastic condition and its activation regulates tumour cells morphology, differentiation and proliferation [Markwalder, R., Reubi, J. C., Gastrin-releasing peptide receptors in the human prostate: relation to neoplastic transformation. Cancer Res 59 (1999) 1152-1159]. These findings encourage the search of bombesin derivatives,

which could be radiolabeled and used for imaging and/or delivering a cytotoxic radiation dose to prostate tumor cells.

A universal bombesin ligand, D-Tyr-Gln-Trp-Ala-Val-ß-Ala-His-Phe-Nle-NH_2 (6–14), has been developed by Mantey et al. [Mantey, S. A., et al., Discovery of a high affinity radioligand for the human orphan receptor, bombesin receptor subtype 3, which demonstrates that it has a unique pharmacology compared with other mammalian bombesin receptors. J Biol Chem 272 (1997) 26062-26071] and Pradhan et al. [Pradhan, T. K., et al., Identification of a unique ligand which has high affinity for all four bombesin receptor subtypes. Eur J Pharmacol 343 (1998) 275-287], which has high affinity to all of the bombesin receptor subtypes. Based on this universal ligand, Saudi Arabia has synthesized the new bombesin analogue DOTA-Glu-BNU (DOTA-Glu-Phe-Glu-Trp-Ala-Val-ß-Ala-His-Phe-Nle-NH_2). The sequence contains the spacer Glu-Phe-Glu. The rational of introducing negatively charged aminoacids in the sequence was to enhance the renal excretion and reduce abdominal uptake. The sequence has already been synthesized and will be distributed among the participating countries (Saudi Arabia, Argentina, Brazil, Syria, Macedonia, Poland, India, Austria, Hungary, Italy-Rome, Italy-Milano) for further evaluation during the second part of this CRP.

3. SPECIFIC RESEARCH OBJECTIVES FOR THE SECOND PART OF THE CRP

The results presented by the various participant groups indicate that the primary objective of this CRP, which was aimed at developing an easy-to-use, freeze-dried, sterile kit formulation for labelling the antibody Rituximab with ^{177}Lu and ^{90}Y, might become feasible. The procedures for antibody's conjugation and labelling have been precisely defined and this will pave the way for designing the final kit formulation. Hence, a major expected outcome for this CRP still remains the development of efficient technologies for producing pharmaceutical kit formulations for radiolabelling antibodies with trivalent therapeutic metallic radionuclides.

Similarly, the extension of methods employed for labelling somatostatin-derived peptides with ^{177}Lu and ^{90}Y to other peptides having potential application for cancer therapy continues to constitute another research elevant objective of the present project. In this respect, the development of a freeze-dried kit formulation is less challenging than for antibodies, the most critical issue being to find a peptide derivative having optimal properties for targeting specific cancer cells.

4. WORKPLAN FOR THE SECOND PART OF THE CRP

4.1. Rituximab

The protocols reported in the next paragraphs describe the detailed procedures that should be employed by all research groups for conducting studies on the labelling of Rituximab with ^{177}Lu and ^{90}Y. Since no significant differences have been found between the various bifunctional chelating agents **1**-**5**, the compounds **1** and **2** pictured in Fig. 1 have been selected for subsequent studies. The laboratory in Rome, Italy, will perform the chemical characterization of the resulting BFCA-MoAb conjugates as well as the determination of their imunoreactivities. Then, the various research groups will use these well-characterized antibody conjugates for labelling and stability studies. In a similar way, the Italian laboratory will determine the immunoreactivity of the resulting radiolabelled BFCA-MoAbs. Animal studies for assessing the biodistribution properties of the radiolabelled conjugates will be carried out by the laboratory in Hungary. The bulk of data collected from these studies will provide the experimental basis for the subsequent development of a convenient freeze-dried kit formulation according to the main objective of the CRP.

4.2. Bombesin

4.2.1. Delivery of the peptide and radiolabelling

Saudi Arabia has synthesized a bulk of 15 mg of peptide and will initially send 1-2 mg to five participating countries (India, Brazil, Argentina, Italy-Rome, Italy-Milan). In a more advanced stage of the evaluation, the peptide will be delivered also to the other participating countries. Radiolabelling with high-specifc-activity ^{177}Lu will be performed and the labelling conditions, in terms of peptide amount, labelling buffer, temperature, incubation and pH, will be optimized to obtain high labelling efficiency (India). It is expected that a standard radiolabelling protocol will be elaborated, which can be thereafter followed by all participating countries.

4.2.2. Stability studies in vitro

In three different centers, stability studies in solution and in serum will be performed with ^{177}Lu-DOTA-Glu-BNU ((DOTA-Glu-Phe-Glu-Trp-Ala-Val-ß-Ala-His-Phe-Nle-NH_2 = DOTA-Glu-BNU). Results will provide some initial information on the stability of the radiolabel and against enzymatic degradation.

4.2.3. Binding studies and cell uptake studies

Depending on the availability of different BBN receptor positive cell lines (PC-3 prostate cancer, MDA-MB-231 or MCF-7 breast cancer) saturation assays will be perfomed with ^{177}Lu-DOTA-Glu-BNU. Additionally, internalisation studies will be performed in the same cell lines to evaluate the receptor specific uptake into the cells. Brazil will perform these studies on ^{111}In-labelled DOTA-Glu-BNU. In this way, the results can be compared with results available for other two bombesin analogues, BZH3 and $BEYG_5N$, which have been previously evaluated. A complementary in vitro binding assay study will be performed by the laboratory in Rome, Italy, using the new 'Ligand Tracer ' automated device.

Based on these experimental results, it will be possible to assess whether the selected bombesin peptide shows a promising biological profile for a further in vivo evaluation. Only after completing this initial evaluation, the peptide will be send also to the other participating countries (Syria, Macedonia, Poland, Austria, Hungary).

4.2.4. In vivo biological studies

In vivo studies will be performed in normal mice, including also metabolite studies, as well as in a mouse xenograft model. The obtained results will be again compared with previous results obtained with BZH3 and $BEYG_5N$. These in vivo investigations will definitely demonstrate if the new radiolabelled bombesin analogue exhibits lower abdominal uptake and higher renal excretion associated with high tumour uptake and prolonged retention. It is expected that these biodistribution studies will allow to draw clear conclusions on the clinical potential of the peptide DOTA-Glu-BNU.

4.2.5. Development of a kit formulation

After completion of all evaluation steps, Macedonia will formulate a freeze-dried, sterile and pyrogen-free kit formulation to be conveneintly employed for subsequent investigational studies in human subjects.

5. PROTOCOLS

5.1 Conjugation of Rituximab to *p*-SCN-Bn-DOTA (1) and *p*-SCN-Bn-DTPA (4)

5.1.1. Reagents

- Rituximab (10 mg/mL)
- *p*-Isocyanatobenzyl-DOTA (*p*-SCN-Bn-DOTA, **1**), *p*-isocyanatobenzyl-DTPA (p-SCN-Bn-DTPA, **2**) (Macrocyclics)
- PBS buffer (0.1 M, pH 8.5)
- PBS (0.05 M)/NaCl (0.15 M) buffer (pH 7.2)
- NH_4OAc (0.5 M)
- NH_4OAc (0.05 M)
- Sodium hydroxide (1.0 M)
- Hydrochloric acid (1.0 M)
- Metal-free water (typical resistance, 18 mΩ per cm)

5.1.2. Equipment

- Ultrafiltration device [i.e., Vivaspin, Amicon, (MWCO 30 kDa)]
- Calibrated pH meter
- Metal-free plastic test tubes
- Refrigerated centrifuge
- UV spectrophotometer
- MALDI-TOF MS

5.1.3. Reagent setup

- *PBS buffer (0.1 M, pH 8.2)*. Dissolve 17.9 g of $Na_2HPO_4 \times 12H_2O$ in 500 mL of water (A). Dissolve 6.8 g of $NaH_2PO_4 \times H_2O$ in 500 mL of water (B). Add 23.0 mL of solution B to solution A (resulting pH = 8.5).
- *Mixture of PBS (0.05 M)/NaCl (0.15 M)*. Dissolve 17.9 g of $Na_2HPO_4 \times 12H_2O$ in approximately 1 liter (dm^3) of water. Add 8 g of NaCl and 5 mL of HCl (2 M). Mix vigorously and check that the pH is between 7.0 and 7.2.

5.1.4. Preparation of Rituximab for conjugation

{Caution! If Rituximab is not provided at a density of 10 mg/mL or it is not dissolved in a 0.1-M PBS buffer solution, please follow the steps given below.}

(1) Pipette the required amount of protein solution (1.1 mL) into the ultrafiltration tube
(2) Dilute to 2 mL with 0.1-M PBS buffer, pH 8.5.
(3) Spin the tube in a centrifuge so that the protein solution volume is reduced by a factor of 2–3.
(4) Discard the ultrafiltrate and repeat steps (2) and (3).
(5) Spin the tube until the volume reduces to 1 mL or less (if you have less than 1 mL bring it up to 1 mL with PBS (0.1 M, pH 8.5).
(6) Measure the absorbance of a protein sample at 280 nm in a UV spectrophotometer. Calculate the protein concentration (mg/mL) using the value of 1.4 $(mg/mL)^{-1}$ for the molar extinction coefficient.

5.1.5. Conjugation reaction

[1] Calculate the amount of BFCA required to give a 20-fold molar excess over the amount of Rituximab.
[2] Dissolve a sufficient quantity of DOTA (**1**) (or DTPA, **2**) bifunctional ligand in 0.1-M PBS (pH 8.5) to give a concentration of 10 mg/mL. Calculate the volume of the resulting solution required to fit with point [1] and add this to the protein solution.

[3] Adjust the pH with NaOH (1 M) and/or HCl (1 M) using a pH meter to a final pH of 8.5. Mix immediately and incubate at 4 ° C overnight with gentle shaking.

5.1.6. Purification of the conjugate using ultrafiltration devices

1) Rinse the ultrafiltration tube with ammonium acetate, pH 7 (0.05 M for storage or 0.5 M for immediately labelling).
2) Pipette the reaction mixture into a tube.
3) Dilute the mixture to 2.0 mL with 0.05-M or 0.5-M ammonium acetate, pH 7.
4) Spin the tube in a centrifuge so that the protein solution volume is reduced by a factor of 2–3.
5) Repeat steps 3) and 4) until the absorbance in the ultrafiltrate set at 280 nm is nearly zero (meaning that no more free chelating agent is being eliminated from the solution).
6) Increase the volume to obtain a protein concentration of 5 mg/mL.
7) Determine the concentration of the conjugate using some well-established method for measuring protein concentration (i.e., Bradford, BCA).

5.1.7. HPLC analysis of the immunoconjugate

Immuconjugates should be characterized by SE-HPLC. Briefly, about 20 µL of the conjugate MAb is injected into a size-exclusion column using a mixture composed of NaCl (0.9%):NaN_3 (0.05%) as mobile phase. The flow rate is maintained at 1 mL/min and the elution is monitored by UV spectrophotometer at 280 nm. The area under the immunoconjugate peak should span more than 95% of the total area.

5.2. Labelling of the Rituximab conjugate

5.2.1. Radiolabeling

An aliquot of the appropriate radionuclide (^{177}Lu, ^{90}Y; 1000 MBq in 10–15 µL) in HCl (0.05 M) is added to 100 µL of NH_4OAc buffer (0.5 M) at pH 7.0 followed by 200 µL (1 mg) of the corresponding immunoconjugate. Then, the reaction mixture is incubated at 42 °C for 1 h, or at room temperature for 30 min, when *p*-SCN-Bn-DOTA (**1**) or *p*-SCN-Bn-DTPA (**2**) are used, respectively.

5.2.2. Quality control

In order to scavenge any free radiometal for further quality control, a solution of DTPA or EDTA (0.01 M, pH = 6.0) corresponding to 1/10 of the sample volume withdrawn for the analysis, is added to the reaction vial and the mixture is incubated for 15 min at room temperature. Radiochemical purity is determined by thin-layer chromatography on SG-ITLC plates (Pall Corporation, USA), using 10% (w/v) ammonium acetate:methanol (1:1) as mobile phase. In these conditions, the radioimmunoconjugate remains at the origin and the radiometal-DTPA (EDTA) complex migrates to the front.

SE-HPLC can be also employed using an instrument equipped with a size exclusion HPLC column and one online radio-detector, and following the same procedure described above for the immunoconjugate.

When required, radiolabeled conjugates are purified from unbound radiometal by ultracentrifugation.

The acceptance limit for RCP is ≥ 95%.

5.3. Chemical characterization and immunoreactivity of BFCA-MoAb conjugates

5.3.1. Protein characterisation by matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF)

Both characterization of the conjugated BFCA-MoAb and determination of the average number of BFCA attached to each antibody molecule can be performed by Matrix-Assisted Laser Desorption Ionization time-of-flight (MALDI-ToF) mass spectrometry. A representative procedure can be outlined as follows. A volume (10 μL) of the solution of the conjugated BFCA-MoAb is diluted (1:10) with a matrix solution of 3,5-dimethoxy-4-hydroxycinnamic acid [10 mg/mL dissolved in a mixture of acetonitrile (50%)/TFA (1%) (Sigma)] to a concentration of about 10 pmol/μL. An aliquot (1-2 μL) of the final solution is applied to the sample target prior to insertion into the high vacuum chamber of a mass spectrometer (Voyager-De MALDI-ToF, Applied Biosystems). Operational conditions for the MALDI-ToF apparatus are set as follows: Mode of operation, linear; Polarity, positive; Acceleration voltage, 20000 V; Delayed extraction time, 100 nsec; Acquisition mass range, 140000-170000 Da.

5.3.2. In vitro competitive binding assay

To test the in vitro binding affinity of radiolabelled Rituximab for specific receptors, a competitive binding assay can be performed on CD20-positive Burkitt lymphoma-derived cell lines, RAJI [Schaffland AO, Buchegger F, Kosinski M, Antonescu C, Paschoud C, Grannavel C, Pellikka R, Delaloye AB (2004) 131I-rituximab: relationship between immunoreactivity and specific activity. J Nucl Med 45:1784–1790]. The cells are maintained in a RPMI 1640 culture medium (Sigma-Aldrich) supplemented with 10% heat-inactivated foetal calf serum (Gibco), 1% L-glutamine (Gibco), 1% antibiotics (penicillin and streptomycin, Gibco), in a 5% CO_2 humidified incubator at 37 °C. Cell viability and cell count are determined by trypan-blue assay using a haemocytometer.

For binding experiments, triplicate samples of the radiolabelled-Rituximab are incubated at increasing concentrations (from 0.05 to 50 nM) with 10^5 cells either alone or in the presence of a 100-fold molar excess of the unlabelled antibody to saturate the specific receptors on the cells. After 2 hours of incubation, cells are harvested by centrifugation (9,000g for 3 min) and washed two times with complete RPMI 1640 culture medium. Cells and supernatants are then collected in different vials and counted separately for radioactivity in a single-well gamma counter (Gammatom, Italy). The plot of specific binding is generated as the difference between total binding and nonspecific binding. A Scatchard analysis is finally performed using GraphPad Prism Version 5.00 Software (GraphPad Software, Inc.) to determine the dissociation constant (K_d).

5.3.3. Automated in vitro binding assay

The in vitro binding assay illustrated above is usually performed manually. Since several methods are available, it is worthy only to recommend that experiments should be always carried out in triplicate or quadruplicate (both with and without displacement). Alternatively, these experiments can be performed automatically using a new instrument called ‘Ligand Tracer’, which has been designed to perform simultaneously several binding assays and to follow them in real time. The instrument is composed of a rotating device on which a Petri dish can be placed (Fig. 2). Cells are grown in a target area on the dish and then incubated with the radiopharmaceutical. The dish is then started to rotate and the activity associated with the target area measured by a detector. The device is connected to a computer in order to follow the binding assay in real time and to collect and plot data using dedicated software. This instrument allows performing a fast preliminary screening of different labelled compounds on different cell lines, in order to provide a preliminary evaluation of the pharmacokinetics and to give insights on the most promising radiopharmaceutical to be used for further experiments. The following tests can be performed with this device: (1) kinetic binding assay to determine the dissociation and association rate constants (off-rate and on-rate) of unlabeled compounds, (2) affinity assay to determine the strength of the ligand-receptor interaction (K_d), (3) competitive binding assay to determine the specificity of the ligand-receptor interaction (4) internalization assay to determine the internalized

fraction and the intracellular retention time. With this apparatus it is also possible to analyze the formation of metabolites in the supernatant.

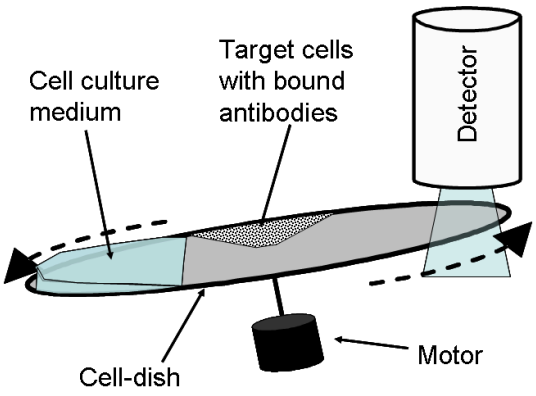

FIG. 2. Experimental setup for automated in vitro binding assay

Similar information can be also obtained by ex-vivo autoradiography on paraffin-embedded tissue samples incubated with the radiolabelled MoAb.

5.3.4. Immunoreactive fraction assay

The assay for determination of the immunoreactive antibody's fraction, carried out by linear extrapolation to conditions representing infinite antigen excess, has been adapted from the method described by Lindmo et al. [Lindmo T, Boven E, Cuttitta F, Fedorko J, Bunn PA Jr (1984) Determination of the immunoreactive fraction of radiolabelled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. J Immunol Methods 72:77–89] with only slight modifications. The immunoreactive fraction assay is performed using a constant concentration of radiolabelled MoAb and serial dilutions of RAJI cells. The cells are washed three times in phosphate-buffered saline (PBS, pH 7.4) and suspended in a cold phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) solution. Radiolabelled MoAb, at a constant concentration of 50 ng/mL in PBS with 1% BSA solution, is added to different amounts of cells (final concentration ranging from 2.6×10^6 to 0.08×10^6 cells/mL). Cells are incubated for 2 hours at 4°C and then washed twice with 500 µL of cold PBS with 1% BSA solution. Then, cell-associated radioactivity is counted in a single-well gamma counter. Data are plotted as a double inverse plot of the applied radiolabelled antibody (Y axis = 1/cpm or nM of radiolabeled MAb) over the specific binding as a function of the inverse cell concentration X axis = 1/cells per mL). In this plot, the origin of the abscissa represents infinite cell concentration, i.e., conditions of infinite antigen excess. All experiments must be performed in triplicate [Malviya G, Anzola KL, Podestà E, Laganà B, Del Mastro C, Dierckx RA, Scopinaro F, Signore A (2012) ^{99m}Tc-labeled Rituximab for imaging B lymphocyte infiltration in inflammatory autoimmune disease patients. Mol Imaging Biol 14:637-646].

5.4. Animal studies on ^{177}Lu/^{90}Y-labelled Rituximab

To complement in vitro measurements on immunoreactivity, cell binding, saturation and dissociation assays, another crucial information is obtained by in vivo evaluation of the biodistribution of the radiolabelled antibody in tumour animal models. These xenograft models are usually produced by inoculation of the same cell lines (RAJI or equivalent) employed for in vitro studies. The preliminary verification of the presence of the target

CD20 antigen on the cell surface by immunohistochemistry or other methods is always considered an essential prerequisite before developing the tumour animal model.

5.4.1. Biodistribution test in normal mice and in nude mice xenografts

Preliminary biodistribution studies in healthy mice (e.g., BALB/C mice) are primarily recommended, though other species (e.g., rats, Guinea pigs, Syrian hamsters) are also feasible. A representative procedure is outlined here. The radiolabelled compound is injected intravenously (usually through a tail vein) into the animals (a minimum of 3 animals per group is always required), with injected activity ranging from 5 to 10 MBq (volume range from 0.1 to 0.2 mL) for each animal. For biodistribution studies with MoAbs, early (2 hours post injection) and late groups (24 hours post injection) are always required, but introduction of additional time points is strongly recommended. After administration, the animals are anaesthetized usually by narcotic gases (isofluran, aether, chloroform) or injectables [pentobarbital-Na (40 mg/bwkg ip), ketamine-HCl (10 mg//bwkg) + xylazine (2 mg/bwkg ip)], then euthanized using either an overdose of injectable narcotic (e.g., 400 mg/bwkgs pentobarbital-Na, ip) or registered veterinary drugs (T-61 inj AUV, Euthanyl inj). Organs, samples (tail, blood, heart, lungs, liver, kidneys, spleen, GI-tract, muscle, bone/femur, brain and tumor) are then removed, washed, dried, scaled and radioactivity measured. Radiopharmaceutical uptake is calculated as percent of injected activity per whole organ or, alternatively, as injected activity per gram tissue.

5.4.2. Imaging studies

Equipment specially designed for laboratory animal imaging (preferably a microSPECT/CT small-animal tomograph) is recommended for imaging studies with β-emitting radionuclides that also decay through γ-emission (e.g., ^{177}Lu), though rats and mice could be conveniently scanned using also conventional cameras for human studies. In this case, the use of a pinhole collimator is strongly recommended if available to achieve a better resolution in small laboratory animals. The imaging study is generally performed both in healthy and xenografted mouse models, but other rodents (e.g., Guinea pig, Syrian hamsters) are also acceptable alternatives. In those centers where imaging can be conducted on larger animals (e.g., healthy Beagle dogs, swines and referred B-cell lymphoma dogs) additional studies on these animal models are also recommended. A common procedure for imaging is to inject a solution (0.1–0.2 mL) of the radiolabelled compound (10–20 MBq) into a mouse. After anesthesia carried out using the methods mentioned above, the sleeping animal can be imaged. Different animals can be used if imaging is started at different time points after injection. A minimal imaging procedure requires collecting ventrodorsal whole body images at 3 and 24 hours post injection, but registration of additional serial images is strongly recommended. Though qualitative imaging provides a suitable picture of the in vivo biodistribution behaviour of the radiolabelled compound, quantitative scintigraphy (conducted by ROI or VOI analysis) should be carried-out whenever possible.

5.5. Radiolabelling and biological evaluation of Bombesin-derived peptides

5.5.1. Radiolabelling with Lu-177

All buffers used for radiolabelling should be prepared with metal free water. Only high specific activity $^{177}LuCl_3$ will be used for radiolabelling. To ~ 20 μg DOTA-peptide 100-200 μL sodium acetate buffer (0.4 M, pH 4.5) or an alternative buffer will be added and the reaction mixture heated at 90° C for 30 minutes. For higher specific activity ^{177}Lu-labelling, lower amounts of peptide can be used.

5.5.2. Determination of radiochemical purity

Instant thin layer chromatography (ITLC-SG) can be applied to determine free lutetium-177, with citrate buffer (0.1 mol/L, pH 5.0) as solvent (R_f of labeled peptide is 0.0 and R_f of free lutetium is 0.9-1.0).
Radiochemical purity (RCP) should also be determined by high performance liquid chromatography equipped with radioactivity detection, using a RP C_{18} column (4.0 x 150 mm, 5 µm), flow rate 1.0 mL/min using a water/acetonitrile/TFA gradient.

5.5.3. Serum stability assay

Prior to incubation in the different test solutions the reaction solution is purified by solid phase extraction on a C-18 SepPak cartridge. The radiopeptide is then incubated at a concentration of ~1000-4000 pmol peptide/mL and incubated for up to 24 hours at RT of 37 °C.

The radiolabelled peptide is added to 1 mL of human or mouse/rat plasma and incubated for 15-30 minutes, 1, 4 and 24 hours at 37 °C. After each time point, an aliquot of each sample is collected, the proteins precipitated with ethanol or ACN (1:1) followed by centrifugation to separate large proteins. The supernatant containing the radiolabelled peptide is diluted with water (1:1) and analysed by HPLC, and the percentage of intact radiolabelled peptide is determined (% intact peptide of each sample per time point). Similar studies have to be additionally perfomed in phosphated buffered saline or saline.

5.5.4. Receptor binding assay

Saturation binding assays with the labelled peptide will be performed on whole adherent PC-3 cells or an alternative cell line. The cells should be seeded in 6-well plates (2.5×10^5/well) and grow in a 5% CO_2 atmosphere humidifying incubator at 37 °C for 24 h. Radiolabelling is performed to a standardized protocol followed by SepPak purification. The obtained solution will be diluted with PBS/1% BSA to give a 4,000 nM solution, followed by serial dilutions from 400 to 1 nM in PBS/1% BSA. The unlabeled peptide (10,000 nM in PBS/1% BSA) is used as the competitor. The cells are washed with culture medium (1% FBS) and culture medium containing 1% v/v FBS and 0.1% (w/v) sodium azide is added to minimize internalization. Subsequently, 150 µL of either PBS/1%BSA (to measure total binding) or 150 µL of competitor (to measure nonspecific binding) will be added. This is followed by the addition of increasing concentrations of radioligand (final concentration 0.1 – 40 nM). All samples will be repeated in triplicate. The plates are incubated at 20°C or RT for 1.5 h, and then washed twice with culture media containing 1 % v/v FBS and 0.1 % (w/v) sodium azide and lyzed with 1 mL of 1 M NaOH for 15 min and collected. Additionally, two washing steps are done with PBS and collected together with the cells. The protein content of three wells can determined using a Protein Assay kit (Biorad Laboratories). Dissociation constant (Kd) and maximum numbers of binding sites (Bmax) will be calculated with nonlinear regression using a dedicated software. Additionally, also cell internalization studies could be performed.

Additionally, an automated in vitro binding assay using the Ligand Tracer can be also performed in the laboratory in Rome, Italy, following the procedure described in section 5.3.3. Complementary information on the biodistribution behaviour can be also obtained by ex-vivo autoradiography on paraffin-embedded tissue samples incubated with the radiolabelled peptide.

5.5.5. Biodistribution studies in tumour-xenograft mice

GRPR–positive xenografts are induced by subcutaneous injection in nude mice of PC-3 cells (2×10^6/mouse), or any alternative receptor positive cell line, suspended in a 0.2-mL volume. Animals are used 15-21 days later when the tumors grow to around 5 mm in diameter.

The radiolabelled peptide and SepPak purified peptide, diluted in saline solution, is injected intravenously into the tail vein of the tumour–bearing mice (n = 3 for each time point). At each selected time points, the mice are anesthetized, sacrificed and blood, tumour, pancreas, kidneys, heart, stomach, spleen, intestine, liver, lung, and muscle are sampled, weighed and counted in a γ-counter along with activity reference standards. The percentage of injected activity per gram of tissue is lastly calculated for each tissue type.

5.6. Quality of ^{177}Lu to be used for radiolabeling

The following reccommendations have to be applied to the production of the ^{177}Lu through neutron irradiation of ^{176}Lu targets to ensure a good quality for the final ^{177}Lu labelling solution.

1) Enrichment of the ^{176}L target should be as high as possible (optimally > 82% enrichment in ^{176}Lu).
2) It is strongly recommended to perform dissolution of the irradiated target using suprapure grade hydrochloric acid. Similarly, suprapure or ultrapure quality water should be always employed.
3) Specific activity should be as high as possible, but not lower than 16 mCi/μg.
4) Recommended radioactive concentration is 1.0 mCi/μL.
5) The amount of the radionuclidic impurity corresponding to the presence of ^{177m}Lu should be kept lower than 200 ppm at the time of production.
6) Acceptable level of other non-radioactive metallic impurities should be set according to specifications for ^{177}Lu solutions reported in the Perkin-Elmer catalog.
7) pH of $^{177}LuCl_3$ solutions should fall into the range 1-4.
8) Preferably sterile (by autoclaving) and pyrogen-free solutions should be supplied (this is mandatory for clinical studies).

6. EXPECTED OUTPUTS

- Freeze-dried, sterile and pyrogen-free kit formulation for labelling the anti-CD20 antibody Rituximab with ^{177}Lu and ^{90}Y.
- Freeze-dried, sterile and pyrogen-free kit formulation for labelling the peptide Bombesin with ^{177}Lu and ^{90}Y.

7. COOPERATION AMONG PARTICIPANTS

Only a few number of cooperative research activities have been established during the first part of the CRP, mostly because all groups have been involved in protocol optimization and this work do not require extensive cross collaborations. Nonetheless, the following collaborations have been pursued. Austria and Argentina are collaborating on the development and biological evaluation of peptidic minigastrin-receptor targeting radiopharmaceuticals. So far, the A431 epidermoid carcinoma cell line stably transfected with the human CCK-2 receptor has been established in Argentina and two minigastrin analogues are currently available for characterization in animal models. Italy (Rome) is actively cooperating with the laboratory in Hungary for conducting biological studies in large animals with new radiolabelled antibodies. India is supplying on demand reactor produced Lu-177 for labelling and biological studies. Saudi Arabia and Argentina will cooperate to investigate the labelling of a new HER2/neu receptor peptide with Lu-177.

According to the workplan, a sharp increase of research collaborations among the different laboratories is expected to occur during the second part of this CRP as requested by the need to share and compare experimental data for developing the final kit formulations.

8. RECOMMENDATIONS

It is envisaged that the following actions might further help the successful accomplishment of the relevant research work planned for this CRP.

- Provide support and assistance for the purchase and distribution to the various groups of chemicals and biologically active compounds required for the preparation of the final radiopharmaceuticals.

- Establish a research contract with a specialized laboratory for conducting both chemical and in vitro biological characterization of the resulting bifunctional ligands (BFCA-MoAbs and BFCA-peptides) and of the radiolabelled conjugates.

9. CONCLUSIONS

The progress of the research work was accomplished according to the workplan proposed during the first RCM of this CRP and detailed in the first Report. Specifically, the rationale of the proposed program was to determine the most appropriate experimental conditions for conjugation and radiolabelling the antibody Rituximab. This information is essential for planning the final development of a lyophilized kit formulation for preparing this therapeutic radiopharmaceutical, which constitutes a key objective of this CRP. The various research groups have provided a sufficient amount of data that strongly support the feasibility of this product and, therefore, suggest that the research plan could be successfully completed during the second part of the CRP.

A parallel program was focused on the selection of a peptide derivative that might constitute a step forward on the route for the discovery of other useful peptide-based radiopharmaceuticals potentially having the same successful clinical applications as somatostatin-derived receptor targeting radiopharmaceuticals. After some initial emphasis on the peptide substance-P, it has been concluded that bombesin-related peptides might offer more advantages, particularly considering the recent development of many structurally related analogs. This conclusion will affect the second part of the CRP since more extensive studies will be devoted to this class of peptidic radiopharmaceuticals.

10. SUMMARY OF COUNTRY PRESENTATIONS

Argentina

In this summary we briefly describe the work carried out in the Radiopharmacy Division during the last year related with the scope of the CRP. The main focus was the conjugation of SCN-Bz-DTPA with Rituximab®, the radiolabelling of this conjugated with locally produced ^{177}Lu and to perform HPLC studies to determine the radiochemical purity. *In vitro* stability test of the product were done using c-DTPA as a tranchelator at different molar ratios. ^{177}Lu-DTPA-benzyl-Rituximab® biodistribution were carried out in normal mice at different time p.i. One freeze dried kit formulation for labelling with ^{177}Lu were developed. High Specific Activity (H.S.A.) ^{177}Lu was obtained for the first time in Latinamerica in an experimental scale production using the direct method from an 82 % ^{176}Lu enriched target. The level of ^{177m}Lu / ^{177}Lu was determined by gamma spectrometry. ^{177}Lu-DOTA-Substance P biodistribution were carried out in normal mice with and without previous arginine infusion in order to quantify its renal protection effect. Internal dose assessment for quantification of this effect in normal mice and extrapolation to standar human patients were made in collaboration with the Dosimetric Group (Nuclear Regulatory Authority). Preliminar studies of ^{177}Lu-DOTA-His2-MG11 in nude mice bearing A431 CCK+ human cells were initiated. As a consecuence of a training period at the Nuclear Medicine Department of

the Insbruck University, one of our fellows participated in the synthesis of two new DOTA-Minigastrin analogues and acquired experience in internalization studies of radiolabelled DOTA-peptides.

Austria

Beside the clinical use of ^{90}Y-DOTA-TOC and ^{177}Lu-DOTA-TATE for treatment of neuroendocrine tumours our department is continuously working on the development of novel radiopharmaceuticals based on other peptide analogues. Within the first year of this project we designed new minigastrin (MG) analogues based on DOTA-MG11, including collaborations with Turkey and Argentina. The new peptide derivatives contain unnatural amino acids in the peptide sequence with the aim to stabilize the peptide analogue against enzymatic degradation.

Another contribution of our department was the further optimization of the clinical preparation of Lu-177 and Y-90 labelled somatostatin analogues. In this respect we have evaluated the possibility to reduce radiolysis and elaborated procedures for the quantification of the total peptide amount in the final preparation. A future activity in this direction will be the optimization of the automated dispensing and infusion of the single dose to the patient.

Brazil

In the field of radiolabelled molecules, substance P (SP), bombesin and Rituximab appear as promising molecules for radiopharmaceutical design, because they can target specifically to receptors present mainly in glioblastoma, prostate cancer and non-Hodgkin lymphoma, respectively. During this project, the DOTA and DTPA-peptides and Rituximab will be radiolabelled with ^{177}Lu, applying our previous knowledge and a method for their ^{90}Y-labelling will be developed. The radiolabelled molecules will be evaluated in preclinical studies in healthy and tumour mice. The radiopharmaceuticals with the best performance after these evaluations will be studied in order to develop a formulation, which should follow the requirements for clinical applications.

China

In this report, the purification of mAb, the conjugation of mAb with CHX-A"-DTPA and p-SCN-Bn-DOTA, the measurement of chelator/mAb ratio in the conjugates using spectrophotometry were described. The factors, which influenced ^{177}Lu labelling of conjugates were studied, and RCPs were analyzed with ITLC and HPLC. The RCPs of labelled conjugates were over 95% after purification with PD-10 column. The in-vitro stability of labelled conjugates in 10mm DTPA, saline, 5% HSA, 10% HSA was investigated. The preliminary biodistribution studies of labelled conjugates in normal mice were also carried out.

Cuba

During the first period of this CRP we have been working in the final setting up of the technology of the ^{90}Sr/^{90}Y electrochemical generator. The module and software are currently working properly. We followed different procedures to establish the best electrochemical parameters for ^{90}Y separation (current or voltage values, time of electrolysis, washing agents, effects of carrier addition and number of electrolysis cycles for purification of ^{90}Y). The mean yield for ^{90}Y has been between 90 and 95%. Nowadays we are establishing the best electrochemical conditions to reach the extremely low level of ^{90}Sr breakthrough of 2 μCi in 1.0 Ci of ^{90}Y.

Molecules of interest to bind to therapeutically useful radionuclides are still monoclonal antibodies and their fragments. In this regard, we continued working on DOTA-NHS conjugation of hR3 (Nimotuzumab) at different experimental conditions in order to obtain labeling efficiencies higher than 95% while keeping the highest degree of immunoreactivity. Special attention was devoted to biological models required to evaluate the radiolabelled conjugate, mainly by in vivo assessment of tumour uptake. Another antibody of interest is the anti CD20 (Rituximab), which was conjugated to DTPA-CHX also using different experimental conditions. We found that radiochemical purity higher than 95% was achieved at a molar ratio of 1:20.

Hungary

The major work carried-out in Hungary focused on in vivo testing of radiopharmaceutical applicant molecules. Elements of a complete biological evaluation system were published in earlier IAEA-coordinated projects. In this present period beyond the known normal and pathological animal distribution-, and radiotoxicological studies we established several double xenografted Nude mice models that are available to image and quantify the specific and non-specific binding of tumor-seeking radioligands. Spontaneously occurring canine tumors (eg.: canine insulinomas and B-cell lymphomas) were also selected and their usefulness proved in therapeutical radiopharmaceutical research. Selection of the referred animal patients based on PET/CT or SPECT/CT whole body hybrid images (^{68}Ga-DOTA-TATE, ^{99m}Tc-HYNIC-TATE, and FDG PET/CT) and immunohistochemical evaluation of biopsy specimens were carried-out. We also performed pilot studies for enhancing the radiopharmaceutical uptake in tumor xenografts by the use of locoregional electromagnetic hyperthermia (Oncothermia®). This complete animal testing method is capable to provide important preclinical data including safety-, kinetic-, excretion-, internal dosimetry-, and efficacy results that are assisting the decision making process for investigators. Hungary still offers its animal testing capacities for all other interesting participating countries.

India

The objective of the present CRP is to develop ^{177}Lu and ^{90}Y-labeled radiotherapeutic agents based on monoclonal antibodies and peptides. Towards this, production of adequately high specific activity clinical grade ^{177}Lu has been augmented and suitable method to separate ^{90}Y from ^{90}Sr has been developed at our Institute. These two radionuclides have been utilized for the preparation of ^{177}Lu/^{90}Y-labeled Reditux® and ^{177}Lu-labeled MabThera® (both Reditux® and MabThera® are commercially available anti-CD20 monoclonal antibody). A cold kit of DOTA-TATE, suitable for the preparation of therapeutic patient dose of ^{177}Lu-DOTA-TATE, has also been developed and evaluated.

Italy (Milan)

We focus on the optimization of the conjugation of three bifunctional chelating agents (BFC) to a monoclonal antibody such as hR3 and Rituximab.
The following procedures were implemmented:

- conjugation at molar ratios 1: 50 and 1:100,
- purification of the conjugated molecules using PD-10 desalting column,
- ^{111}In- radiolabelling of immunoconjugates,
- quality control analysis to calculate the radiochemical purity and also to determine the average number of chelates per antibody,
- stability of labelled products using an excess of DTPA.

In summary, we have developed a conjugation procedure, optimized the QC of the immunoconjugates by checking the number of BFC per molecule of antibody, performed a successful labeling with ^{111}In and checked the stability of the labeled products using an excess of challenging agent.

We conclude that we have become familiar with the basic techniques to obtain an immunoconjugate and we are ready to repeat these experiments for further evaluations by the member states.

Italy (Rome)

In the framework of the CRP on "Development and preclinical evaluation of therapeutic radiopharmaceuticals based on ^{177}Lu and ^{90}Y labeled monoclonal antibodies and peptides" we proposed to participate as a specialized center for pre-clinical in vitro screening of kit formulations of ^{177}Lu-labelled-mAb/peptides synthesized by other laboratories participating in this IAEA coordinated project. Over the past years we developed know-how on studying the biological properties of candidate radiopharmaceutical performing routinely different in vitro quality controls and binding assays. Therefore, we applied our experimental protocol to test HYNIC- and DTPA-

conjugated Rituximab labeled with ^{99m}Tc in order to test its biological properties. We found that the Rituximab molecule can efficiently be conjugated to both bifunctional chelators and radiolabelled, retaining the dissociation constant of the native mAb. On the basis of the obtained results we propose again our center for centralized in vitro QC and small animal studies.

Moreover, we carried on our experiments on labeled superagonist rhTSH analogue TR1401 to develop an imaging and therapeutic radiopharmaceutical for thyroid cancer metastasis. We performed in vitro and in vivo binding studies on TSHR+ve and TSHR-ve cell lines that proved the specificity of labeled TR1401 for TSH receptor. We also imaged several mice with TSHr +ve tumors and, in collaboration with Dr Balogh from Hungary we also imaged cats with spontaneous tumors to verify the specificity of this new tracer. Next year we will continue our experiments in mice and cats and dogs.

Macedonia

The purpose of this investigation was to introduce a technology for the production of ready-to-use cold kit formulations for the labelling of conjugated DOTA-Rituximab and peptide-based ligands (Substance-P) with Lu-177 and Y-90, and to standardize the methods for synthesis and conjugation. In a parallel study, the biological properties and pharmacokinetic behaviours of radiolabelled DOTA-Rituximab and Substance-P were investigated to compare and determine their toxicities and therapeutic efficacies using model systems comprising both isolated cell cultures and laboratory animals.

A preliminary study on Substance-P (SP) has been pursued with the aim to obtain a peptide derivative suitable for labelling with Tc-99m and Re-188. For this purpose, modified Substance-P peptides (Cys-Cys-SP/PCN for Tc-99m labeling and IsoCys-Cys-SP/PCN for Re-188 labelling) were prepared and labelled with Tc-99m and Re-188 and the resulting characteristics were examined including biodistribution studies in normal mice.

The other part of our work was devoted to establish an efficient freeze-drying procedure for developing a final kit formulation for simple antibody labelling. The procedure for freeze-drying should provide a stable ready-to-use kit formulation, having the same labelling efficiency as established for the freshly prepared laboratory preparation. The freeze-dried formulation should preserve the same immunoreactivity of the antibody before and after conjugation as already observed for the liquid formulation. For this reason, we focused our study on the evaluation of the most important steps required to set up a sutable freeze-drying protocol and, in particular, to estimate the optimal time for lyophilization of the monoclonal antibody during each phase of the process, e.g., (a) pre-freezing, (b) primary drying and (c) secondary drying.

Poland

DTPA- or DOTA-chelated antibodies have been proved to be effective in radioimmunotherapy of cancer after radiolabelling with beta-emitting radiometals. The standardization of methods for conjugation of suitable chelators to monoclonal antibodies and the further pharmaceutical development of these conjugates were planned within the framework of an IAEA coordinated research project aimed at developing radiopharmaceuticals labelled with ^{90}Y or ^{177}Lu. Therefore, our study was focused on the development of a freeze-dried kit formulation based on DOTA-anti-CD20 Rituximab for ^{177}Lu labeling as potential radiopharmaceutical for radionuclide therapy.

Saudi Arabia

The main purpose of this research project was to develop ^{90}Y and ^{177}Lu-labeled bioactive compounds (antibodies and peptides) for targeting of human cancers. A number of peptides have been prepared by solid-phase peptide synthesis according to Fmoc/HBTU chemistry. After radiolabeling with ^{177}Lu radionuclide, these compounds were evaluated for their potential as radiotherapeutic agents. The results of these evaluations are discussed.

Syria

The general scope of the CRP project is the development of peptide- and antibody-based radiopharmaceuticals labelled with ^{177}Lu and ^{90}Y to be applied to tumour treatment. We defined many specific objectives such as: (a) preparation of ^{177}Lu- or ^{90}Y-labeled DOTA-Rituximab as therapeutic radiopharmaceutical for the treatment of non-Hodgkin's lymphomas following two different conjugation approaches using as bifunctional chelating agents (BFCA) the ligands p-SCN-Bz-DTPA and p-SCN-Bz DOTA, and (b) optimization of labelling technique (incubation time, optimal pH, temperature, specific activity) of conjugated-MoAbs and of protocols for developing a kit formulation for radiolabelling Rituximab with ^{177}Lu and ^{90}Y.

Appendix A

Country reports

ARGENTINA

Production of Lu-177 for labelling antibodys and peptides

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Abstract. In this report we briefly describe the work carried out in our laboratory during the first part of the Co-ordinated Research Project (CRP). Its includes new results in the experimental production of High Specific Activity (H.S.A.) ^{177}Lu, determination of ^{177m}Lu /^{177}Lu level , conjugation of SCN-Bz-DTPA with Rituximab®, radiolabelling of DTPA-Rituximab with locally produced ^{177}Lu, i*n vitro* stability test of ^{177}Lu- DTPA-Rituximab, challenge of ^{177}Lu- DTPA-Rituximab with DTPA and preparation of DTPA-Rituximab in a kit form. In reference to ^{177}Lu-DOTA-peptides we evaluated the *in vivo* renal protecction effect of the arginine infusion in ^{177}Lu-DOTA-Substance P injected mice. Dosimetrical calculations and extrapolation to normal patients were done using the OLINDA program. Regarding to the other radiolabelled peptide, preliminary ^{177}Lu-DOTA-His2-MG11 biodistribution studies in nude mice with A431 CCK+ human cells were initiated. Collaboration with the Austrian counterpart was established through a training period at the Nuclear Medicine Department of the Insbruck University, in the field of new DOTA-Minigastrin analogues synthesis and internalization studies.

1. Introduction

Radioimmunotherapy (RIT) and Peptide Receptor Radionuclide Therapy (PRRT) are two important fields of the Nuclear Medicine which require the development of new targeted radiotherapeutics particularly for the treatment of B-cell lymphomas, malignant brain tumour and neuroendocrine tumours. During the last 15 years we were working at the Radiopharmacy Division in the research and development of new radiopharmaceuticals for cancer radionuclide therapy based on different beta negative emitters. The recent local experimental production of H.S.A ^{177}Lu, a low-energy β-emitter, allow us the possibility to obtain high radiochemical purity radiopharmaceuticals such us ^{177}Lu-benzyl-DTPA-Rituximab, ^{177}Lu-DOTA-Substance P and ^{177}Lu-DOTA-His2-MG11 for *in vivo* evaluation in mice. The collaboration with the austrian counterpart make possilbly to initiate ^{177}Lu-DOTA-His2-MG11 biodistribution studies in nude mice with A431 CCK+ human cells (gifted by the austrian group). ^{177}Lu-DOTA-Substance P dosimetrical calculations and extrapolation to normal patients were done in order to quantify the arginine renal protecction effect.

2. Objective

The main objective of the present work was to obtain ^{177}Lu-Bz-DTPA-Rituximab and Bz-DTPA-Rituximab in a freeze-dried kit form for labelling with the local produced ^{177}Lu. The second aim was to evaluate ^{177}Lu-DOTA-His2-MG11 in tumored nude mice and to quantify the arginine renal protecction effect in normal mice injected with ^{177}Lu-DOTA-Substance P.

3. Description of the work done during the first year of the CRP

Our activities during the first year were divided into eigth parts:

3.1. S.A. improvement in ^{177}Lu experimental production.

During the period 2011 (2012), 7 (2) targets were irradiated. Total activity produced was 173.82 mCi at the end of bombardment (EOB). During the last part of 2011 and 2012 the irradiation time of the target was extended from 3 to 4 irradiation cycles in order to obtain a higher ^{177}Lu S.A. than before [1]. The target irradiation was done at the RA-3 maximum thermal neutron flux (neutron trap position). The level of ^{177m}Lu / ^{177}Lu was 105 ppm for a 14.84-day irradiation. Calculated ^{177}Lu S.A. obtained irradiating the enriched target 82.0 % ^{176}Lu (provided by the Agency) was approximately 24.1-27.88 Ci/mg at EOB. The highest S.A. obtained at the labelling time was 23.98 Ci/mg. This ^{177}Lu S.A. was enough for radiolabelling biomolecules such as DOTA-SP and monoclonal antibodies with high radiochemical yield.

3.2. Conjugation of SCN-Bz-DTPA chelator to MoAb anti CD20

The conjugation of p-SCN-Bz-DTPA (Macrocyclics, USA) to 10 mg of Rituximab (c= 10 mg/mL) (Roche, Argentina) were carried out at a molar ratio 20:1 (pH 9, 37 ºC) during 1 h.
The product was purified by centrifugation using a Vivaspin 4 filter (Sartorius, Argentina). The preparation of a freeze-dried kit of Bz-DTPA-Rituximab for instant labeling with ^{177}Lu was described in reference [2].

3.3. Labelling of DTPA-Rituximab using ^{177}Lu locally produced

1 mg of Bz-DTPA-Rituximab (+ 150 µl of ammonium acetate 1M pH 7) was radiolabelled with 2,0-2,5 mCi of $^{177}LuCl_3$ (S.A. = 15.7-18,3 mCi/µg of Lu-176). The incubation conditions were 30 min. at 37 ºC. The radiochemical purity of the ^{177}Lu-Bz-DTPA-Rituximab determined by HPLC-Gel Permeation was 99.5 % and the S.A. was 2 mCi/mg of protein. .
In the case of the freeze-dried kit of Bz-DTPA-Rituximab labelled with ^{177}Lu (S.A. = 9.21 mCi/µg), the radiochemical purity was between 95-99 %.

3.4. In vitro stability of ^{177}Lu-Bz-DTPA-Rituximab

The radiolabelled product was challenged with c-DTPA using a molar ratio from 1:1 up to 1:1000, and measuring radiochemical purity at 1 and 24 h. The percentages of ^{177}Lu transchelation for each molar ratio were 5 % and 2% at 1 h, and 8 % and 5 % at 24 h, respectively.
The results of ^{177}Lu transchelated corrresponding to the product obtained from the frezee-dried kit were 9.1 % and 17.2% at 1 h respectively and 69.4 % and 99.9 % at 24 h respectively.

3.5. In vivo stability of ^{177}Lu-Bz-DTPA-Rituximab

Biodistribution in normal mice for the ^{177}Lu-Bz-DTPA-Rituximab are listed in Table 1.

TABLE 1. Biodistribution of ^{177}Lu-Bz-DTPA-Rituximab expressed as % of Injected activity/ per gram of tissue (% I.A./g).

Time p.i.	2h	24h	48h
Blood	24,25 ± 3,22	14,26 ± 3,20	12,25 ± 2,29
Liver	4,35 ± 0,68	5,44 ± 0,33	5,24 ± 1,76
Spleen	3,16 ± 0,30	2,85 ± 0,32	2,54 ± 0,22
Kidney	4,77 ± 0,41	3,57 ± 0,41	3,33 ± 0,55

Stomach	0,92 ± 0,16	1,22 ± 0,13	0,58 ± 0,11
Intestine	0,89 ± 0,20	0,65 ± 0,11	0,49 ± 0,18
Lungs	6,00 ± 0,70	4,11 ± 0,38	3,42 ± 0,26
Fémur rigth	1,54 ± 0,06	1,83 ± 0,21	1,77 ± 0,28
Fémur left	1,57 ± 0,08	1,86 ± 0,15	1,74 ± 0,27

The results showed a low accumulation in fémur (less than 2 % I.A./g) at 48 h p.i. It means that the product do not present *in vivo* unstability by ^{177}Lu transchelation.

3.6. Preliminary results of ^{177}Lu-DOTA-His$_2$-MG11 biodistribution in a nude mouse with and without A431 CCK+ human cells at 1 h p.i.

DOTA-His2-MG was labelled with ^{177}Lu locally produced (S.A. 14.5 mCi/µg) by the well-established technique. One nude mouse was inoculated subcutaneously with 10^6 A431 cells CCK+. After 10 days the tumored mouse and a normal mouse were injected with 20 µCi of ^{177}Lu-DOTA-His$_2$-MG11. Biodistribution were carried out at 1 h p.i. and the results expressed as % of injected activity (Fig.1) as a consecuence of a relative big tumor mass (2.46 g). The accumulation in the tumor at 1 h was as 3.38 % I.A.

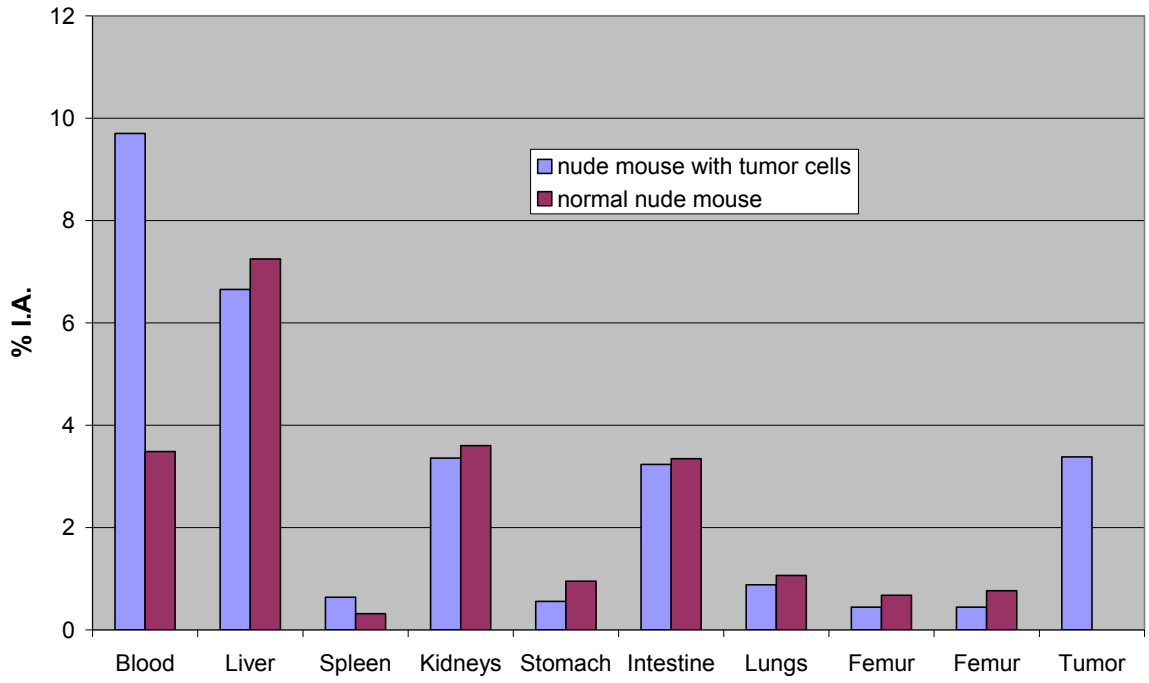

FIG.1. Biodistribution of ^{177}Lu-DOTA-His$_2$-MG11 in a normal nude mouse (red bars) and in a nude mouse bearing A431 CCK+ cells (blue bars) at 1 h p.i.

3.7. Biodistribution ^{177}Lu-DOTA-SP in normal mice with and without arginine infusion

In a previous work [3], ^{177}Lu-DOTA-Substance P was evaluated in normal mice and dosimetrical calculations were done in order to extrapolate the dose to patients.

The renal protection effect of arginine in normal mice injected with ^{177}Lu-DOTA-Substance P was studied. Statistical comparison between treated and non treated mice were done [4]. There was statistically significant difference in the average ^{177}Lu-DOTA-SP renal accumulation beetween the two groups without and with arginine (Fig. 2) at 30 and 120 min. p.i.

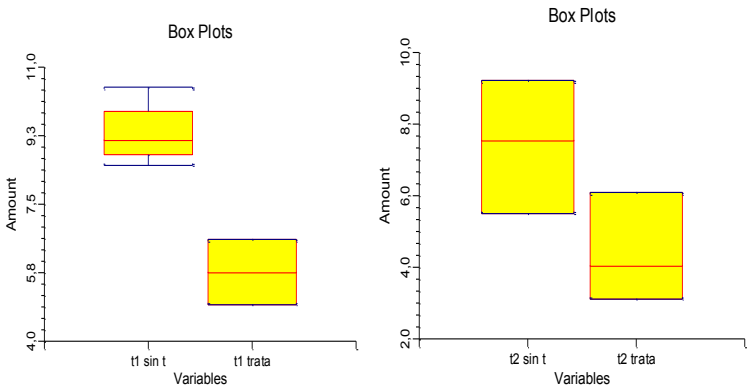

FIG.2. ^{177}Lu-DOTA-SP renal accumulation: comparison between non treated (left box) and arginine treated mice (rigth box) at 30 and 120 min p.i respectively.

However, there was not statistically significant difference beetween the average of two groups (Fig.3) at 360 min. p.i.

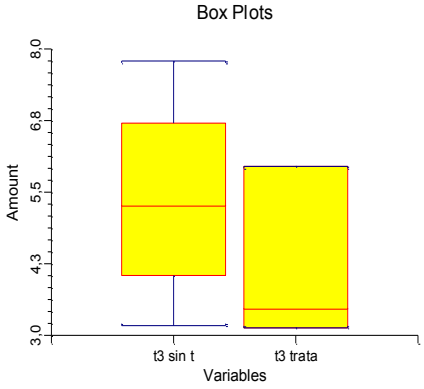

FIG.3. ^{177}Lu-DOTA-SP renal accumulation: comparison between non treated (left box) and arginine treated mice (rigth box) at 360 min p.i.

3.8. Internal dose assessment in ^{177}Lu-DOTA-SP injected mice with and without arginine and extrapolation to standar human patients

Absorbed dose from ^{177}Lu-DOTA-SP per unit of I.A. for two conditions, with and without prior administration of arginine, in organs of the NIH mouse, adult female (56,9 kg) and adult male (76,7 kg) is showed in Table 2. All this work were made in collaboration with the Dosimetric Group (Nuclear Regulatory Authority) [5].

TABLE 2. Absorbed dose from ^{177}Lu-DOTA-SP per unit of I.A (mGy/MBq) for NIH mouse with and without arginine and extrapolation to standard women and man.

Organs	Absorbed Dose (mGy/MBq)					
	with Arginine			without Arginine		
	Mouse	Woman	Man	Mouse	Woman	Man
Kidneys	115.07	1.04	1.16	139.9	1.15	1.59
Liver	8.47	0.06	0.06	4.61	0.02	0.03
Lungs	17.05	0.02	0.02	14.57	0.06	0.05
Stomach	5.64	0.01	0.01	9.67	0.05	0.05
Spleen	27.70	0.09	0.07	14.85	0.05	0.05
Intestine	1.81	0.10	0.10	4.66	0.22	0.22
Bone Marrow	0.57	0.01	0.01	0.51	0.01	0.01

The Maximum tolerated activity (MAT) is the calculated maximum activity of ^{177}Lu-DOTA-SP that can be administered to patients (Table 3) without exceeding the tolerance dose of the kidneys (20 Gy).

TABLE 3. Maximum tolerated activity (MBq/kg) of ^{177}Lu-DOTA-SP for women and man with and without arginine.

MAT for case with Arginine		MAT for case without Arginine	
Woman (MBq/kg)	Man (MBq/kg)	Woman (MBq/kg)	Man (MBq/kg)
338	234	306	170

4. Description of the work proposal for the rest of the CRP

4.1. Conjugation of SCN-Bz-DOTA and 1B4M-DTPA (2-(4-iso-tio-ciano-bencil)-6-metil-DTPA) to MoAb anti CD20

This study will be conducted according to the established protocols and compared with the results obtaine by the other participating groups.

4.2. Preparation of a freeze-dried kit of DOTA-Rituximab and 6-CH3-DTPA-Rituximab for instant labelling with ^{177}Lu and ^{90}Y

Freeze-dried kit formulations for antobody's labelling will be checked for stability and labelling efficiency.

4.3. In vitro binding studies of the different radiolabelled products

The *in vitro* binding studies of the products will be performed, in order to determine its biological activity after the labelling procedure. In the case of radiolabelled Rituximab the target will be, B-cells from human leucopheresis and Raji cells from Burkit`s lymphoma.

4.4. Biodistribution and dosimetrical studies of the different radiolabelled products

Biodistribution and dosimetrical studies will be carried out in normal mice and in tumored nude mice (if available) for ^{90}Y-SCN-Bz-DTPA-Rituximab, ^{177}Lu(^{90}Y)-SCN-Bz-DOTA-Rituximab [6] and ^{177}Lu(^{90}Y)-1B4M-DTPA-Rituximab. We will extend the research to other potential candidates as a monoclonal antibodies and new universal bombesine-DOTA labelled with ^{177}Lu depending on its availability.

4.5. Biodistribution and dosimetrical studies of the ^{177}Lu(^{90}Y)-DOTA-Minigastrin analogues

In the near future we expect to radiolabel two new DOTA-Minigastrin analogues (gifted by the Austrian counterpart) with ^{177}Lu and ^{90}Y, to carry out internalization studies in A431 CCK+ cells and *in vivo* evaluation studies in tumored nude mice bearing A431 CCK+ cells. If the result were successfull we will initiate dosimetrical pre-clinical studies.

5. Collaboration among participants

Collaboration with the Austrian counterpart was established through a training period at the Nuclear Medicine Department of the Insbruck University, in the field of new DOTA-Minigastrin analogues synthesis and internalization studies.

ACKNOWLDEGEMENTS

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AUSTRIA

Radiolabelling of biomolecules with ^{90}Y and ^{177}Lu and development of novel targeting strategies

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1. Introduction

The use of radiolabelled antibodies and peptides for targeted radionuclide therapy of cancer is an established procedure in nuclear medicine. Beside the clinical use of ^{90}Y-DOTA-TOC and ^{177}Lu-DOTA-TATE for treatment of neuroendocrine tumours our department is continuously working on the development of novel radiopharmaceuticals based on other peptide analogues.
Within the first year of this project we designed new minigastrin (MG) analogues based on DOTA-MG11. The new peptide derivatives contain unnatural amino acids in the peptide sequence with the aim to stabilize the peptide analogue against enzymatic degradation. The new derivatives have been radiolabelled with In-111 for a first preclinical evaluation in vitro.
Another contribution of our department was the further optimization of the clinical preparation of Lu-177 and Y-90 labelled radiopeptides. In this respect we evaluated the possibility to reduce radiolysis and elaborated procedures for the quantification of the total peptide amount in the final preparation.

2. New minigastrin analogues based on DOTA-MG11

Under the premise that the radiolabelled MG analogues developed so far are not suitable for clinical application, we have investigated new derivatization strategies. Our current research efforts are focused on the development of alternative peptide analogues with improved pharmacokinetics and targeting properties. Native MG contains a methionine residue in the C-terminal receptor specific sequence –Trp-Met-Asp-Phe-NH_2. This poses the risk of oxidation when a heating step is involved in the radiolabelling step. We have shown that oxidation of methionine results in loss of receptor affinity (1). For the substitution of methionine with other amino acids we found a retained cell internalization and tumour uptake for substitution with norleucine, whereas substitution with isoleucine again resulted in loss of receptor affinity (1,2). It is known that linear peptide analogues are rapidly metabolized by peptidases in vivo. Classical strategies to improve the stability of peptides are d-amino acid substitutions, modification of N- and C-terminus (N-acetylation and C-amidation), replacement of labile amino acids and cyclization. Cyclization was already successfully used for the metabolic stabilization of radiolabelled somatostatin (3) and RGD (=Arg-Gly-Asp) (4) analogues. With a cyclic MG analogue based on MG11 we were however able to obtain only a partial stabilization against degradation (5). In collaboration with Turkey we have determined the enzymatic cleavage sites for a number of MG analogues analyzing the metabolites formed after incubation in serum using MALDI-TOF mass spectrometry (6). Based on the obtained results we were able to design new alternative stabilization strategies (see Figure 1).

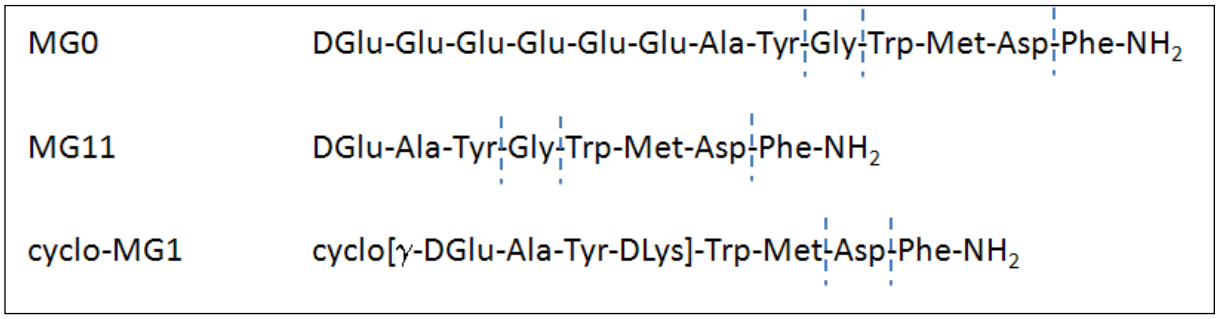

FIG. 1. Cleavage site of different MG analogues characterized by MALDI-TOF MS.

As at the C-terminal receptor binding sequence we found a major cleavage site between Asp and Phe-NH_2 we focused on the modification in this particular region of the peptide sequence. Additionally, we have investigated substitution possibilities for Met which is sensitive to oxidation in heating steps required for radiolabelling. We initially synthesized two peptide analogues, DOTA-MGS2 where Phe in position 8 was substituted with (N-Me)Phe and DOTA-MGS4 where Met in position 6 was additionally substituted with (N-Me)Nle.

The radiolabelling conditions were investigated using three different buffer systems: 1) the Polatom kit containing 50 mg ascorbic acid pH 4.5, 2) 0.4 M ammonium acetate pH 5.5, 3) 0.2 M 2-(N-morpholino)ethanesulfonic acid (MES) pH 5.5. The best radiolabelling yield was obtained by using 100 µl MES buffer together with 30 µg peptide and 50 µl ^{111}In-chloride solution and heating at 95°C for 25 min. Met-oxidation was avoided by addition of 4 mg seleno-methionine. For in vitro characterization experiments the radiolabelling mixture was subjected to solid phase extraction on a SepPak Light cartridge (Waters, Milford, USA).

The determination of the distribution coefficient at pH 7.4 in a 1:1 mixture of phosphate buffered saline and octanol showed a similar log D value for ^{111}In-DOTA-MG11 (-3.55 ± 0.23; n=6) and ^{111}In-DOTA-MGS2 (-3.46 ± 0.19; n=6), while ^{111}In-DOTA-MGS4 (-2.49 ± 0.06; n=6) showed a somewhat higher lipophilicity. The protein binding as determined by incubation in fresh human serum at 37°C and Sephadex G-50 size-exclusion chromatography (MicroSpin, GE-Healthcare Life Sciences, Vienna, Austria) up to 24 h after incubation resulted to be higher for both ^{111}In-DOTA-MGS2 and ^{111}In-DOTA-MGS4 in comparison with ^{111}In-DOTA-MG11 (see Figure 2).

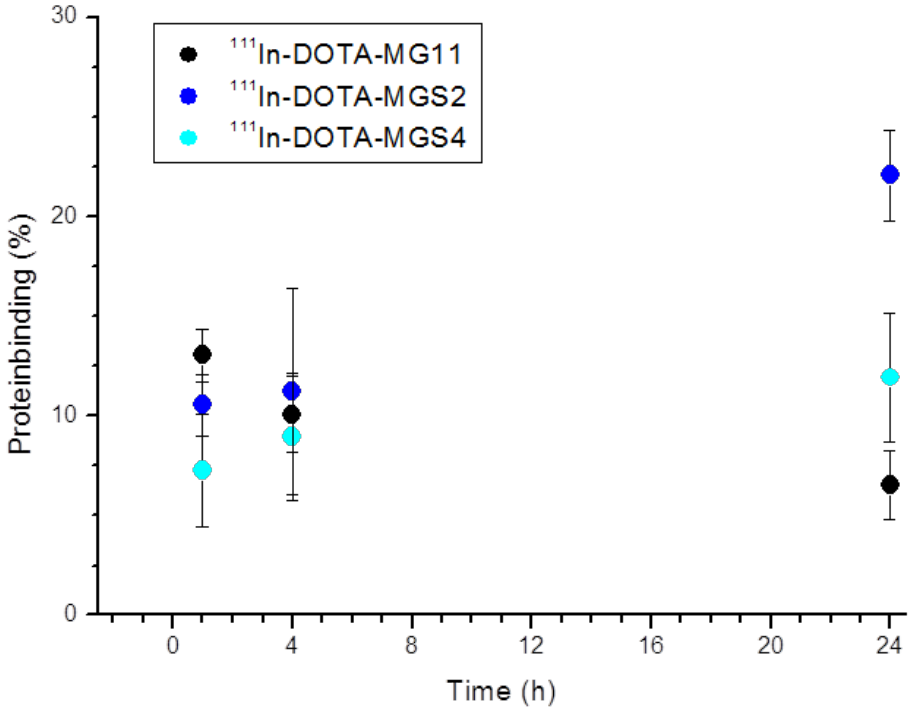

FIG. 2. Protein binding of ^{111}In-DOTA-MGS2 and ^{111}In-DOTA-MGS4 in comparison with ^{111}In-DOTA-MG11.

The stability of the radiolabelled peptides was investigated by incubation in fresh human serum at a concentration of 4000 pmol peptide/mL at 37°C for up to 24 h. Degradation of the radioligands was assessed by radio-HPLC after precipitation with ACN and centrifugation at 2000 g for 2 min (centrifuge 5424, Eppendorf AG, Germany).

As shown in figure 3, ^{111}In-DOTA-MGS2 and ^{111}In-DOTA-MGS4 showed a much higher resistance against enzymatic degradation in comparison with ^{111}In-DOTA-MG11. Even 24 h after incubation still more than 80% of the intact radiopeptide was present, whereas for ^{111}In-DOTA-MG11 this value was only 12% confirming the low stability of this peptide analogue.

In competitive receptor binding assays on AR42J cells and A431 cells transfected with the human CCK-2 receptor (A431-CCK+), using [^{125}I]Tyr^{12}-Gastrin I and increasing concentrations of DOTA-peptide, DOTA-MGS2 (IC50 25-45 nM) showed an impaired binding affinity in comparison with DOTA-MG11 (IC50 2.5-5.5 nM), whereas DOTA-MGS4 again showed a good binding affinity (IC50 5.0-5.5 nM). In accordance with the results of the binding affinity experiments, ^{111}In-DOTA-MGS4 showed a cell uptake of >10% of the total radioactivity added in both cell lines which was comparable with ^{111}In-DOTA-MG11, while ^{111}In-DOTA-MGS2 showed a much lower uptake of less than 5%.

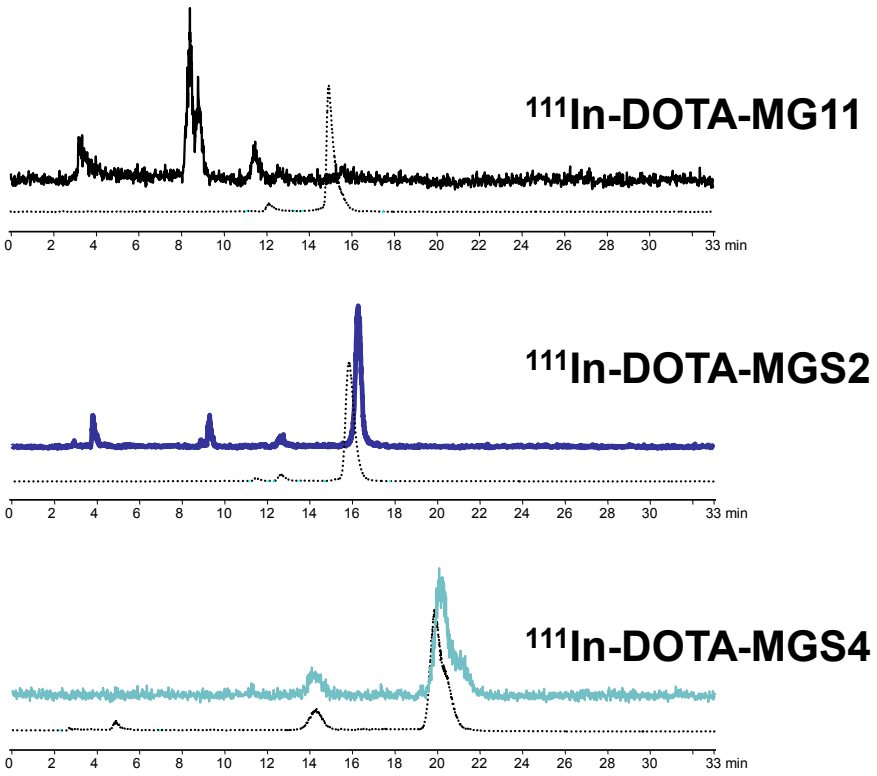

FIG. 3. Stability in human serum of ^{111}In-DOTA-MGS2 and ^{111}In-DOTA-MGS4 in comparison with ^{111}In-DOTA-MG11 (dotted line: radiochemical purity after preparation; solid line: radiochromatogram after 24 h incubation in human serum).

In collaboration with Argentina two other peptide derivatives were synthesized. DOTA-MGS6 beside substitution in position 8 shows the substitution with the respective D-amino acid of Trp in position 5, and DOTA-MGS7 shows an additional D-amino acid substitution of Tyr in position 3. Unfortunately, DOTA-MGS6 according to MALDI-TOF MS after the purification at the end of synthesis resulted to be completely oxidized, only the mass corresponding to the Met-sulfoxide was detected. The peptide analogue has to be subjected to reduction of Met-sulfoxide before performing a further characterization in vitro. DOTA-MGS7 labelled with In-111 showed a very good stability against enzymatic degradation. However, in cell uptake studies performed with both cell lines no specific uptake could be obtained. The reason for this might be the conformational changes introduced by the two D-amino acid substitutions resulting in loss of receptor affinity.

3. Optimization of clinical formulations

The preparation of ^{90}Y-DOTA-TOC and ^{177}Lu-DOTA-TATE is routinely performed in an automated cassette based synthesis module (Eckert&Ziegler, Berlin, Germany). After the reaction the solution is subjected to solid phase extraction on a SepPak tC18-cartridge (Waters, Milford, USA) and sterile filtration. The final solution contains 8-24 GBq Lu-177 or 4-10 GBq Y-90, ~200-600 µg DOTA-peptide, 2-3% ethanol and 0.9% sodium chloride solution to obtain a total volume of 20 ml. The original reaction solution contains ascorbic acid which is stabilizing the solution against radiolysis. As the final solution does not contain a stabilizing agent we performed stability studies at room temperature, 5°C and -20°C on the final preparation showing that in these conditions the final formulation was stable only for 4 h. After 1 day storage only 70% of the intact radiopeptide was present and after 3 days the stability dropped to 16-32%. To see if we are able to stabilize the final formulation against radiolysis we added a stabilization buffer containing ascorbic acid and sodium chloride, adjusted to pH 5. The

storage at the different storage conditions was tested for up to 3 weeks and the obtained results are summarized in Table 1.

TABLE 1. Stability of ^{177}Lu-DOTA-TATE in stabilization buffer at different storage conditions

Storage at	1 day	3 days	7 days	15 days	21 days
RT	94.2	88.9	86.0	74.9	yellow solution
5°C	94.6	91.7	93.1	91.3	81.6
-20°C	94.3	94.7	93.5	93.2	87.2

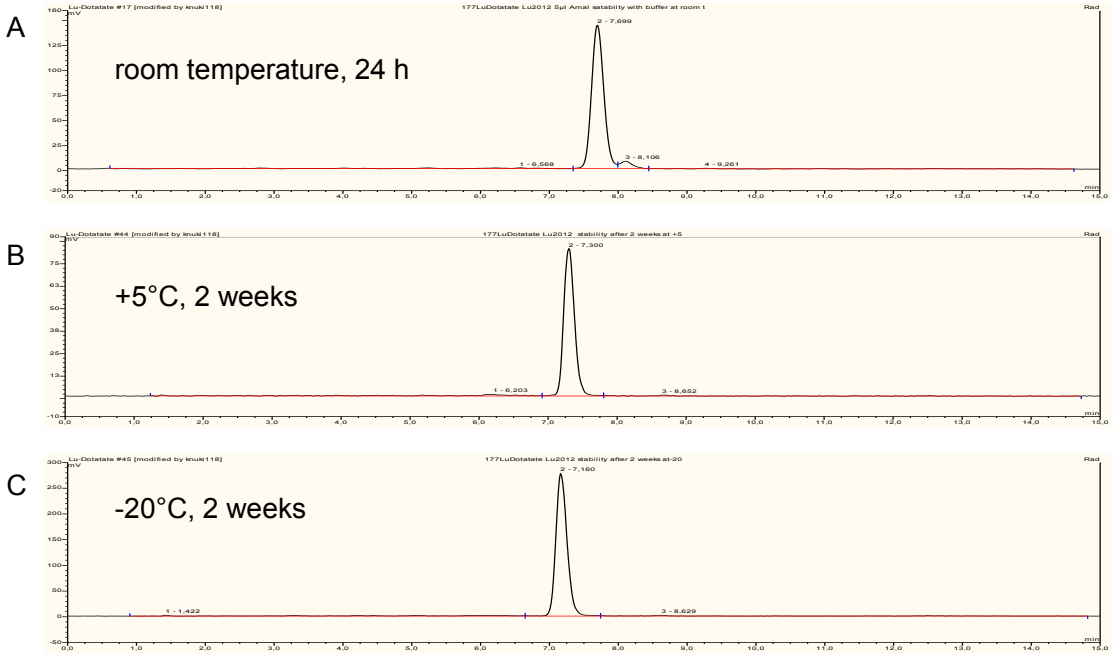

FIG. 4. Radiochromatograms of ^{177}Lu-DOTA-TATE in stabilization buffer at different storage conditions: A) room temperature 24 h, B) +5°C 2 weeks, C) -20°C 2 weeks.

By adding the stabilization buffer we were able to stabilize the final formulation at room temperature for 24 h and for 2 weeks when the product was stored at +5°C or -20°C. In figure 4 the radiochromatograms are shown for the different storage conditions at these time points.

For the determination of the radiochemical purity we are using a combination of HPLC and TLC. The HPLC-gradient is based on a gradient system of acetonitrile/water/TFA and was optimized to allow the identification of different DOTA-peptides radiolabelled with different radiometals. Beside identification also the quantification of the total DOTA-peptide amount in the final preparation is an important parameter in the quality control. The problem in this regard is that for the HPLC analysis only a very small aliquot containing less than 1 µg DOTA-peptide is used. Based on a UV standard curve using a range of 0.1 to 0.8 µg DOTA-peptide we calculated the peptide amount of different preparations of ^{90}Y-DOTA-TOC and ^{177}Lu-DOTA-TATE. All found UV-peaks corresponding to the unlabelled und labelled peptide were included in the calculation. When comparing the total

peptide amount used for the radiolabelling and the peptide amount calculated from the UV-HPLC analysis we found a deviation of ±20-30% allowing only a rough estimation of the peptide amount in the final formulation.

4. Conclusions

Within the first year of the CRP we have proceeded in developing new stabilized MG analogues showing promising results in vitro supporting further studies. Furthermore, we continuously improve our technology for the preparation and application of ^{177}Lu- and ^{90}Y-labelled peptides for targeted radionuclide therapy, focussing on the automation of the radiolabelling process and optimization of the quality assurance. A future activity in this direction will be the optimization of the automated dispensing and infusion of the single dose to the patient.

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BRAZIL

Preclinical evaluation and formulation of ^{177}Lu and ^{90}Y-labelled peptides and monoclonal antibodies for cancer therapy

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1. Introduction

The Radiopharmacy Department of the Nuclear and Energy Research Institute in São Paulo, Brazil (IPEN-CNEN) produces radiopharmaceuticals for Nuclear Medicine including ^{99}Mo-^{99m}Tc generators, lyophilized kits for labelling with technetium-99m, iodine-131 (solution and capsules), iodine-123, gallium citrate (^{67}Ga), thallium chloride (^{201}Tl), and some labelled molecules, including $^{131/123}$I-MIBG, ^{131}I-lipiodol, ^{153}Sm-EDTMP, $^{153/90}$Y-Hydroxiapatate, ^{177}Lu-DOTATATE, ^{111}In-DTPA-Octreotide and ^{18}F-FDG.

The success in the area of somatostatin receptor-positive tumor targeting with diagnostic and therapeutic radionuclides [1] has stimulated research toward radionuclide targeting of alternative receptor systems overexpressed in tumors. Considering this, promising results have been obtained by IPEN group in the field of development of novel peptides for tumor radionuclide therapy (bombesin and substance P) and methods for antibody conjugation to chelating group and labelling with ^{177}Lu. Further investigations should be done applying the peptides and antibody labelled with ^{177}Lu and ^{90}Y.

In designing radiometal-based radiopharmaceuticals for cancer treatment, important factors to consider are half-life, mode of decay, cost and availability of the radionuclide and the chemical and biological properties of the labelled molecule. In the field of radiolabelled molecules, substance P (SP), bombesin and Rituximab appear as promising molecules for radiopharmaceutical design, because they can target specifically to receptors present mainly in glioblastoma, prostate cancer and non-Hodgkin lymphoma, respectively.

We have developed ^{177}Lu-labelling protocols for DOTA-SP [2] and DOTA- bombesin derivatives [3,4] and procedures for Rituximab conjugation to DOTA chelators followed by ^{177}Lu-labelling. High radiochemical purity radiopeptides were obtained and high stability under storage at 4° C was observed, especially when stabilizers were applied.

During this project, the DOTA and DTPA-peptides and Rituximab will be radiolabelled with ^{177}Lu, applying our previous knowledge and a method for their ^{90}Y-labelling will be developed. The radiolabelled molecules will be evaluated in preclinical studies in healthy and tumour mice and also in *in vitro* tumor cells binding assays. The radiopharmaceuticals with the best performance after these evaluations will be studied in order to develop a formulation, which should follow the requirements for clinical applications.

2. Objective

The general scope of this project is the development of peptides and antibody-based radiopharmaceuticals labelled with ^{177}Lu and ^{90}Y to be applied in tumour treatment.

The specific objectives of the project are: (I) Development of a radiopharmaceutical based on Substance P for brain tumours radionuclide therapy; (II) development of a radiopharmaceutical based on Anti-CD20 for non-Hodgkin lymphoma radionuclide therapy; (III) development of a radiopharmaceutical based on Bombesin for prostate and breast tumours radionuclide therapy.

3. Methods and Results

3.1. Development of a radiopharmaceutical based on Substance P for brain tumours radionuclide therapy

We have studied the ^{177}Lu-radiolabelling and stability of DOTA-Substance P-Met^{11} (DOTA-SP-Met^{11}) [2]. Preliminary studies were done in order to establish the ideal labelling conditions for obtaining the highest yield of labelled DOTA-SP-Met^{11}. All reagents of these experiments were prepared with Chelex 100 treated free metal water. DOTA-SP, 0.4 M sodium acetate buffer (0.2 mL, pH 4.5) and $^{177}LuCl_3$ in 0.05N HCl (specific activity 871 – 920 GBq/mg; IDB, Holland) were heated at different temperatures (70 – 90° C) for different time intervals (15 - 30 minutes). Good results were obtained (RP ≥ 99%) at 80^{o}C for 30 minutes or 90^{o}C for 15 to 30 minutes. Radiochemical purity was also superior than 99% when 92.5 to 185.0 MBq of $^{177}LuCl_3$ reacted with 10 µg of DOTA-SP-Met^{11}and decreases progressively from 96.8 to 74.7%, when using higher specific activities, 25.9 and 40.7 MBq/µg, respectively.

During the first period of this CRP, this labelling protocol was applied to compare DOTA-SP-Met^{11}with DOTA-SP-Nle^{11}, a derivative of DOTA-Substance P in which the last amino acid (methionine) was replaced by Norleucine (Nle), especially in the field of stability under oxidation during labelling. DOTA-SP-Nle^{11}labelled with ^{177}Lu was submitted to preclinical evaluations in mice and cell binding studies.

Additionally, DOTA-SP-Met^{11} and DOTA-SP-Nle^{11} were labelled with itrium-90 and the stability of the preparations was evaluated, using gentisic acid as stabilizer.

3.1.1 Study of reaction parameters for labelling DOTA-SP-Nle^{11} with ^{177}Lu and analysis of oxidation during labelling

The following labelling parameters of DOTA-SP-Nle^{11} were evaluated: time reaction, temperature, mass of peptide and activity. The parameters defined as standard condition were 10µg of peptide; 74 MBq (2.0 mCi) of ^{177}Lu; 90°C; 30 min; 350 rpm.

To analyse the oxidation during labelling, DOTA-SP-Nle^{11} was radiolabelled with ^{177}Lu using the standard condition. The amino acid methionine was also introduced in the labeling reaction (6mg/mL), in order to investigate the influence in the oxidation of the labelled peptide in comparison with the labeling of SP (Met^{11}) with ^{177}Lu.

Figure 1 shows the HPLC profile of ^{177}Lu-labelled DOTA-SP-Met^{11} (A) and DOTA-SP-Nle^{11} (B) and the influence of the Methionine in the reaction medium in oxidation and stability of the preparations.

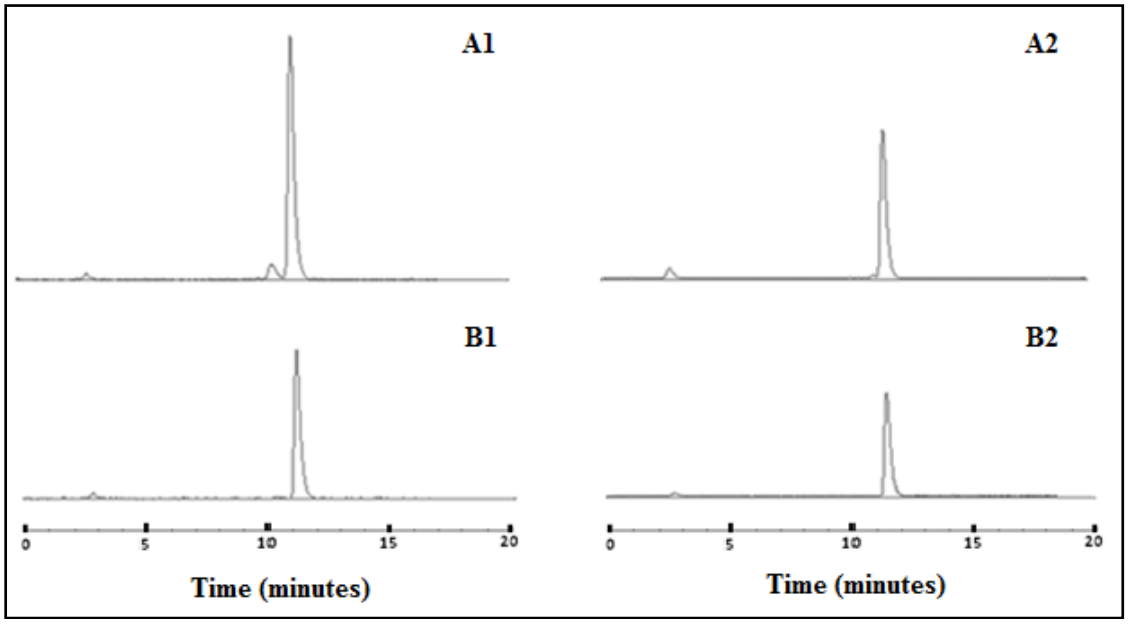

FIG. 1. HPLC profile of ^{177}Lu-labelled DOTA-SP-Met^{11} (A) and DOTA-SP-Nle^{11} (B) with and without methionine (Met) in the reaction medium. ***A1:*** *SP(Met^{11}) without Met in reaction;* ***B1:*** *SP(Nle^{11}) without Met in reaction;* ***A2:*** *SP(Met^{11}) with Met in reaction;* ***B2:*** *SP(Nle^{11}) with Met in reaction.*

The labelling of DOTA-SP-Met^{11} with and without methionine in the reaction medium, resulted in different radiochemical species, as demonstrated in HPLC profile A (RT 10.3 and 11.05 minutes), probably related to the oxidised and non-oxidised form of the labelled peptide, respectively, considering the oxidation of the Met^{11} residue in the protein. When DOTA-SP-Nle^{11} was labelled with ^{177}Lu, the influence of methionine in the reaction was not observed and only one radiochemical form was observed in HPLC profiles (B). As expected, the substitution of Met^{11} for Nle^{11} prevented the oxidation of the labelled peptide.

3.1.2 Study of stability of ^{177}Lu –DOTA-SP-Nle^{11} in human serum and after storing under freezing and refrigeration and in the absence or presence of stabilizers

Stability studies were performed after labelling 100 µg de DOTA-SP-Nle^{11} with 1480 MBq (40 mCi) of ^{177}Lu at 90°C, 30 min; 350 rpm and final volume of 130 µL. Aliquots of 3 mCi of the preparation were incubated in human serum at 37°C for 1, 4 and 24 hrs. The remaining activity was divided in two aliquots and incubated in freezer (-20°C) and refrigerator (2-8°C) for 48 and 168 hours, respectively. Radiochemical purity was determinated by TLC (Table 1) and also by HPLC to the samples stored in refrigerator and freezer.

Stability studies were also performed after labelling 20 µg de DOTA-SP(Nle^{11}) with 333 MBq (9 mCi) of ^{177}Lu at 90°C, 30 min; 350 rpm, and 130µL of final volume, with the addition of ascorbic acid or gentisic acid as stabilizers (6mg/mL) immediately after labelling. An aliquot of 88.8 MBq (2.4 mCi) of the preparation was incubated in human serum for 1, 4 and 24 hrs and the remaining activity was divided equally and stored under refrigeration or freezer. Radiochemical purity was determinate by TLC (Table 1) and also by HPLC to the samples stored in freezer and refrigerator.

TABLE 1. Stability of ^{177}Lu –DOTA-SP-Nle^{11} under different conditions and stabilizers: % RCP determined by TLC.

STABILITY - % RCP DETERMINED BY TLC	
TIME	SERUM (37°C)

(min)	Without stabilizer	With Ascorbic Acid	With gentisic acid
0	97.9 ± 0.6	98.8 ± 0.3	98.2 ± 0.1
1	96.6 ± 0.2	97.9 ± 0.5	98.8 ± 0.1
4	98.5 ± 0.4	98.4 ± 0.1	98.8 ± 0.1
24	98.0 ± 0.1	98.0 ± 0.1	97.8 ± 0.3
TIME (min)	FREEZER (-20°C)		
	Without stabilizer	With Ascorbic Acid	With gentisic acid
0	97.9 ± 0.6	98.8 ± 0.3	98.2 ± 0.1
24	96.1 ± 0.1	97.0 ± 0.8	97.3 ± 0.7
TIME (min)	REFRIGERATOR (2-8°C)		
	Without stabilizer	With Ascorbic Acid	With gentisic acid
0	97. 9 ± 0.6	98.8 ± 0.3	98.2 ± 0.1
24	77.5 ± 2.5	89.3 ± 0.1	92.9 ± 0.2
48	73.9 ± 0.2	86.6 ± 0.5	91.2 ± 1.4
96	57.3 ± 0.2	88.6 ± 0.7	92.1 ± 0.7
120	58.6 ± 0.5	85.7 ± 0.5	89.5 ± 0.7

3.1.3 Biodistribution of ^{177}Lu –DOTA-SP-Nle^{11} in Balb-c *mice*

Biodistribution studies were performed in Balb-c healthy mice, after intravenous administration of 1.67 MBq (45 μCi)/100μL of the labelled peptide (3 animals for each time). The animals were sacrificed at 1, 4 and 24 hours after administration and the organs were removed, weighted and the radioactivity determined in an automated gamma counter. Blood samples were collected for each time. The results were expressed as percent of injected activity (%IA) per gram of organ (Table 2).

TABLE 2. Biodistribution of ^{177}Lu –DOTA-SP-Nle^{11} in *Balb-c* mice in %IA/g

	% IA/g (n=3)		
ORGAN	1 Hour	4 Hours	24 Hours
Blood	0.294 ± 0.018	0.047 ± 0.025	0.012 ± 0.003
Heart	0.187 ± 0.053	0.014 ± 0.005	0.008 ± 0.003
Lungs	0.360 ± 0.070	0.080 ± 0.005	0.021 ± 0.006
Pancreas	0.136 ± 0.030	0.49 ± 0.016	0.011 ± 0.002
Spleen	0.305 ± 0.069	0.092 ± 0.015	0.035 ± 0.010
Stomach	0.207 ± 0.070	0.073 ± 0.016	0.033 ± 0.010
Liver	0.129 ± 0.040	0.197 ± 0.033	0.061 ± 0.013
Kidneys	3.031 ± 0.547	1.391 ± 0.270	0.605 ± 0.138
Intestines	0.479 ± 0.089	0.356 ± 0.075	0.173 ± 0.027

Muscle	0.110 ± 0.025	0.011 ± 0.002	0.004 ± 0.001
Bone	0.655 ± 0.139	0.291 ± 0.088	0.524 ± 0.121
Brain	0.022 ± 0.002	0.005 ± 0.001	0.005 ± 0.001

3.1.4 Labelling of DOTA-SP-Nle^{11} with ^{90}Y and stability studies

DOTA-SP Nle^{11} was labelled with Y-90 (Nordion, Canada), using similar conditions employed for ^{177}Lu. The stability of the preparations were evaluated after 24 and 48 hours under freezing and in the presence of gentisic acid by TLC and HPLC.

TABLE 3. Labelling of DOTA-SP-Nle^{11} with ^{90}Y: 50 ug of peptide, 370 MBq (10 mCi) of $^{90}YCl_3$; 30 minutes, 90^{o}C; 350 rpm – stability study with gentisic acid (n = 2).

Labelling conditions	Radiochemical Purity (%)		
	Immediately	24 hours	48 hours
With gentisic acid as stabilizer (6mg/mL)	97.4 ± 0.3	98.7 ± 0.7	98.1 ± 1.1

Figure 2 shows the HPLC profile of ^{90}Y-DOTA-SP-Nle^{11} 48 hours after labelling represented in the last table.

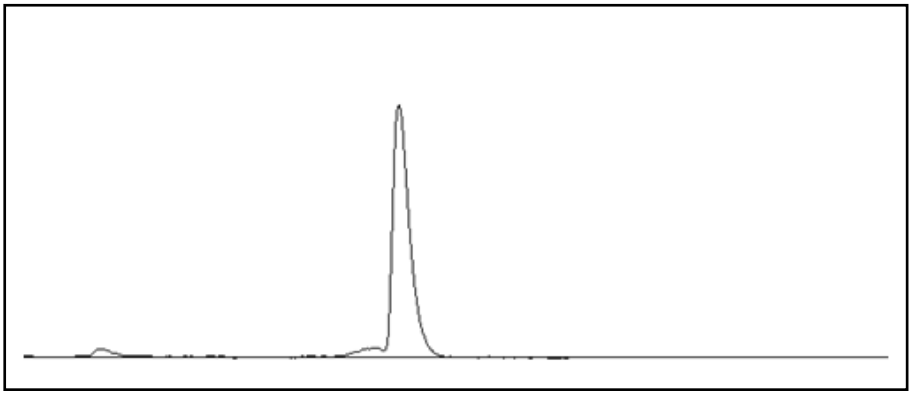

FIG. 2. HPLC profile of ^{90}Y-DOTA-SP-Nle^{11} 48 hours after labelling, with gentisic acid as stabilizer (6 mg/mL) and stored under freezing.

3.2. Development of a radiopharmaceutical based on Anti-CD20 for non-Hodgkin lymphoma radionuclide therapy

We have been studied the DOTA conjugation to Anti-CD20 (Rituximab) antibody and the ^{177}Lu-radiolabelling as a part of the last CRP and the general methods employed are summarized as following.
The pre-purification of the Rituximab solution was studied with Sephadex G-50 columns, dialysis and ultrafiltration tube (Vivaspin, MWCO 30,000, Pharmacia Biotech) using phosphate buffer pH 8.0.

Three different chelators (p-SCN-Bn-DOTA, DOTA-NHS-ester and p- SCN-Bn-DTPA) were conjugated to Anti-CD20 antibody based on procedures previously described, [6-11], using 5-10 mg of antibody previously purified by ultrafiltration device. The reaction was conducted for 1 hour in phosphate buffer pH 8.0, and gently mixing at room temperature and remained for 24 to 48 hours under refrigeration. The molar Ab:chelator ratio employed were 1:50, 1:20; 1:10 and 1:5. Two aliquots of the reaction mixture (about 239 pmol of Ab) were separated to determine the average number of chelators per Ab as previously described [12].

After conjugation, the sample was purified by Sephadex G50 column (1.5 x 20 cm) or ultrafiltration to change buffers (0.2 M acetate buffer pH 5.5) and remove the excess of chelator.

$^{177}LuCl_3$ (111-148 MBq) was added to the conjugated Ab (0.25 to 5 mg) in 0.2 M acetate buffer pH 5.5 and the resultant solution was heated for 1 or 3 hours at 37°C or 43°C in a termomixer (Eppendorf). An aliquot of 10 mM DTPA was added and allowed to react for 15 minutes at room temperature to bind any free radiometal.

HPLC was performed with molecular exclusion column (BioSep SEC-S 3000) and 0.2M sodium phosphate buffer pH 7.0, flux of 1 mL/min for 25 minutes.

During the first period of this CRP, the conjugation parameters was studied, specially Ab:chelator molar ratio and the labelling parameters of this antibody with ^{177}Lu to achieve a standard protocol for preparation of this new radiopharmaceutical. I*n vitro* studies with CD20 positive tumor cells (RAJI) was developed in order to investigate the influence of conjugation and labelling conditions in the immunoreactivity of the antibody. Preclinical studies with the labelled antibody were performed in healthy animals.

3.2.1 Conjugation of Anti-CD 20 antibody using DOTA-NHS-ester

A) Study of the purification method

The purification of the Ab after conjugation with the chelating group is an important step in the preparation of an immunoconjugate. The presence of residual chelating groups in the preparation decreases the labelling of the radionuclide to the immunoconjugate. We studied different methods for purification of the immunoconjugate and ultrafiltration tube (Vivaspin, MWCO 30,000, Pharmacia Biotech) presented better results to remove the excess of chelator and also to reduce the final volume to a protein concentration of about 10 mg/mL (that is important to achieve good results in the labelling procedure). The ultrafiltration system significantly saves purification time and has high recovery efficiency when compared to Sephadex gel column purification.

To determine the number of centrifugations cycles using the ultrafiltration tube, necessary to remove the excess of chelator, the absorbance of the ultrafiltrated was determined (280 nm) after each centrifugation cycle (Figure 3). The experiment was repeated for each Ab:chelator molar ratio employed (1:5, 1:10, 1:20 and 1:50). At least seven cycles of centrifugation were necessary to remove all the excess of chelator after conjugation reactions.

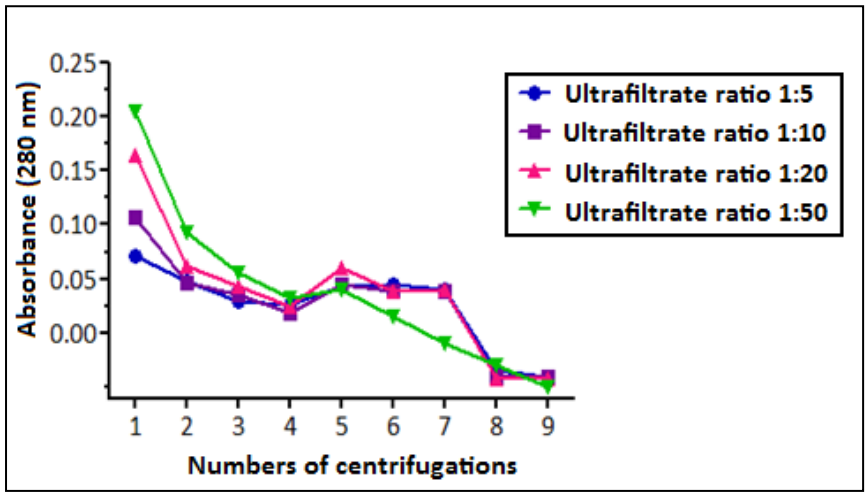

FIG. 3. Absorbance of the ultrafiltrate (280 nm) in each cycle of centriugation (3000 g, 27 minutes under refrigeration).

Figure 4 shows the HPLC profiles of each ultrafiltrate fraction when 10 mg of Ab was conjugated to DOTA using a molar ratio of 1:50. The chromatograms show that the absorbance was reduced to half of value at each cycle of centrifugation and reached almost zero after the last centrifugation cycle.

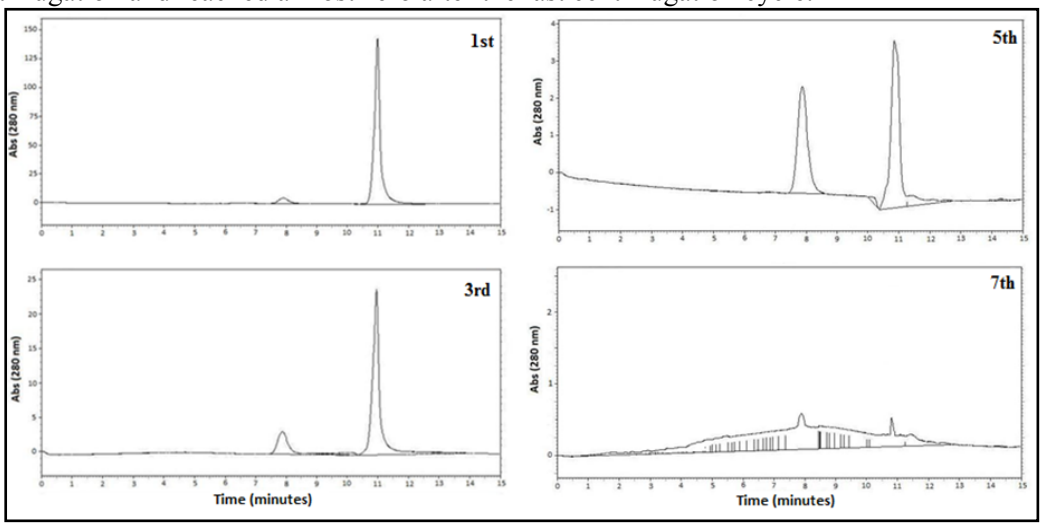

FIG. 4. HPLC profile of ultrafiltrate obtained at each cycle of centrifugation (1st to 7th) in the purification process of the Antibody conjugated with DOTA-NHS.

Another purification protocol was studied in order to reduce the number centrifugation cycles that can damage the antibody structure. This method used a previous purification of the conjugated antibody with Sephadex G25 column, 10 cm (PD-10, Pharmacia) column, followed by two or three cycles of centrifugation. Eighth fractions of 1.0 mL were collected from P-10 column and analyzed by HPLC, so it was possible to identify the fractions corresponding to the conjugated antibody, which corresponds to the first three fractions (not shown).

B) Conjugation using different Ab:DOTA-NHS molar ratios

The conjugation of DOTA-NHS to the Rituximab was performed as previously described, using different Ab:chelator molar ratios (1:5, 1:10, 1:20, 1:50 and 1:100). The final objective was to determine the influence of the number of chelating groups per antibody in the immunoreactivity and stability of the immunoconjugate. The conjugated obtained were analyzed by HPLC after purification by ultracentrifugation.

As expected, the conjugation of chelating groups to Rituximab, increases the molar weight of the antibody and decreases the retention time in the HPLC system employed (using a column of molecular exclusion) (Table 4). The HPLC profiles after purification show only one peak and confirm the efficiency of ultrafiltration as purification method.

TABLE 4. HPLC retention time (RT) for Rituximab, DOTA-NHS-ester e Rituximab-DOTA (conjugated at diferente Ab:chelator molar ratios).

Species	Retention Time (minutes) in HPLC
Rituximab	8.04

DOTA-NHS-éster	10.78
Rituximab-DOTA (1:5)	7.95
Rituximab-DOTA (1:10)	7.93
Rituximab-DOTA (1:20)	7.88
Rituximab-DOTA (1:50)	7.86
Rituximab-DOTA (1:100)	7.80

3.2.2 Labelling of DOTA-NHS-Rituximab with ^{177}Lu

The labelling of DOTA-Rituximab with ^{177}Lu was performed with 1 mg of DOTA-rituximab, 148 MBq (4 mCi) of $^{177}LuCl_3$, 1 hour at 43° C and 350 rpm (Figure 5).

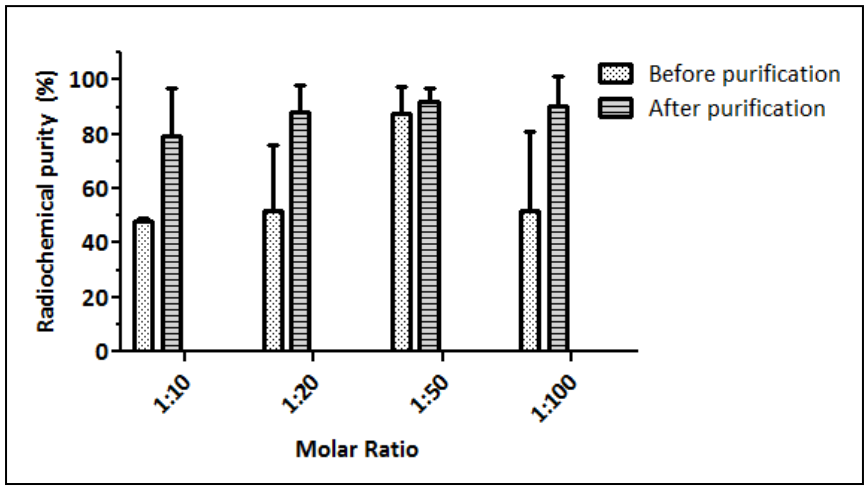

FIG. 5. Radiochemical purity of ^{177}Lu-DOTA-rituximab before and after purification by PD-10 column ($n \geq 3$).

The labelled antibody can be purified using Sephadex G-25 column (PD10, Pharmacia), when radiochemical purity is inferior to 90%. The HPLC profile of purified labelled antibody are showed in the Figure 6.

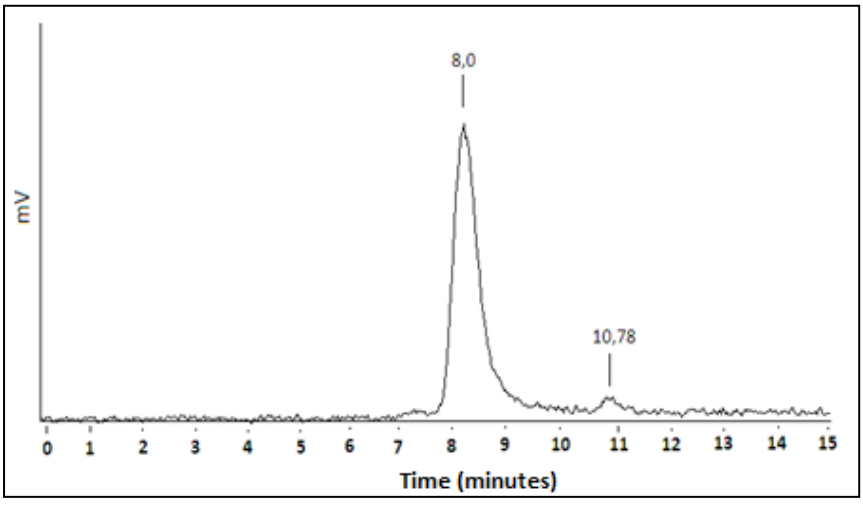

FIG. 6. HPLC profile of ^{177}Lu-DOTA-rituximab after purificaton in Sephadex colum (PD-10 Pharmacia).

The antibody conjugated in the most promising molar ratio (1:50), was radiolabelled with high activity (1480 MBq / 40 mCi) of $^{177}LuCl_3$ (Table 5).

TABLE 5. Radiochemical purity of DOTA-Rituximab labelled with high activity of $^{177}LuCl_3$: 5 mg of DOTA-rituximab, 1480 MBq (40 mCi), 1 hour at 43° C and 350 rpm (n = 2).

Molar ratio	Radiochemical purity (%)
1:50	80.56±0.08

The labelling of DOTA-Rituximab with $^{177}LuCl_3$ was comparatively investigated using the isotope from different suppliers: IBD (870 - 920 MBq/mg) and ITG (non-carrier added) (Table 6).

TABLE 6. ^{177}Lu-DOTA-rituximab labelled with $^{177}LuCl_3$ from IBD and ITG and storage at 2-8 °C. Labelling conditions: 1 mg, 37 MBq (1 mCi) of $^{177}LuCl_3$, 1 hour at 43 °C, 350 rpm (n = 1).

Molar ratio	^{177}Lu- IBD		^{177}Lu- ITG	
	Immediately	24 hs after radiolabelling	Immediately	24 hs after radiolabelling
1:20	77.61±0.004	53.15±0.15	92.80±0.013	91.70±0.004
1:50	91.00±0.062	-	95.33±0.002	94.37±0.005

3.2.3 Biodistribution of ^{177}Lu-DOTA-NHS-Rituximab in Swiss mice

Biodistribution of ^{177}Lu-DOTA-NHS-Rituximab was performed in healthy *Swiss* mice, after intravenous administration of 0.185MBq/0.1mL of the labelled antibody in the tail vein. Aliquots of blood was collected at different time intervals after dose administration and the animals were sacrificed, the organs removed, weighted and the radioactive determined in a gamma counter. The results were expressed as percent injected activity (%IA) per organ or per gram of organ, as following.

TABLE 7. Biodistribution of ^{177}Lu-DOTA-Rituximab (0.185 MBq) in healthy *Swiss* mice (n = 4).

Organ	Time after administration							
	1 hour		4 hours		24 hours		48 hours	
	% IA	% IA /g	% IA	% IA /g	% IA	% IA /g	% IA	% IA/g
Heart	0.90±0.1	7.98±1.2	1.01±0.1	9.09±4.4	0.56±0.1	4.52±0.7	0.64±0.0	5.63±1.3
Lungs	1.61±0.3	8.23±3.0	1.54±0.3	7.01±3.0	1.38±0.1	5.77±1.1	1.47±0.4	5.86±2.1

Pancreas	0.54±0.1	2.81±0.4	0.97±0.2	3.86±1.3	0.54±0.1	2.92±0.4	0.45±0.1	2.09±0.4
Spleen	0.61±0.1	7.51±2.6	0.51±0.1	4.92±2.4	1.58±0.4	8.45±3.2	0.75±0.2	6.74±2.8
Stomach	0.86±0.2	1.18±0.2	1.21±0.1	1.12±0.3	1.28±0.3	1.80±0.3	0.93±0.1	1.06±0.5
Liver	17.32±2.3	13.56±4.0	15.56±1.5	10.09±3.6	19.01±1.4	10.84±2.8	15.54±2.7	10.64±4.6
Kidneys	3.12±0.3	7.47±0.8	3.49±0.4	8.20±0.9	2.66±0.2	5.65±1.3	2.23±0.2	5.19±1.1
Intestines	4.40±0.4	1.51±0.3	6.44±0.3	1.96±0.8	4.97±0.4	1.47±0.3	4.10±0.3	1.29±0.3
Muscle	0.23±0.1	1.09±0.4	0.11±0.0	0.52±0.4	0.30±0.0	1.27±0.2	0.25±0.0	1.16±0.3
Bone	0.27±0.0	3.71±1.0	0.24±0.0	2.53±0.9	0.20±0.0	2.37±0.9	0.21±0.0	2.60±0.4
Brain	0.31±0.1	0.78±0.5	0.40±0.0	0.87±0.1	0.30±0.1	0.68±0.3	0.42±0.0	1.00±0.2

3.2.4 In vitro cell binding of ^{177}Lu-DOTA-NHS-Rituximab

Specific binding assays were conducted for the antibody conjugated at molar ratios with higher radiochemical purity: 1:20, 1:50 and 1:100. The radiolabelled and purified antibody was diluted in medium with 1% of FBS (v/v) with a radioactive concentration of 400 cpm/µL. The non-radiolabeled antibody (4 µM in medium with 1% of FBS v/v) was used as a competitor. The assay was performed by adding different amounts of cells (0.125, 0.25, 0.5 e 1.0 x 10^6 cells, in 0.5 mL of culture medium with 1% of FBS v/v) at conical tubes of 2 mL, followed by addition of 250 µL of medium (1 % FBS) (total binding) or 250 µL of competitor solution (non-specific binding) and 250 µL of diluted radiolabed antibody solution. The conical tubes were incubated by 1.5 hour at room temperature, centrifuged at 5000 *g* by 5 minutes, washed twice with 1 mL of medium with 1 % FBS and analized by gamma counter. The curves of the percentage of total binding, specific binding and non-specific binding, in relation to the number of cells, were constructed using the statistical program GraphPad Prism ® 5.00. The experiment was performed in triplicate.

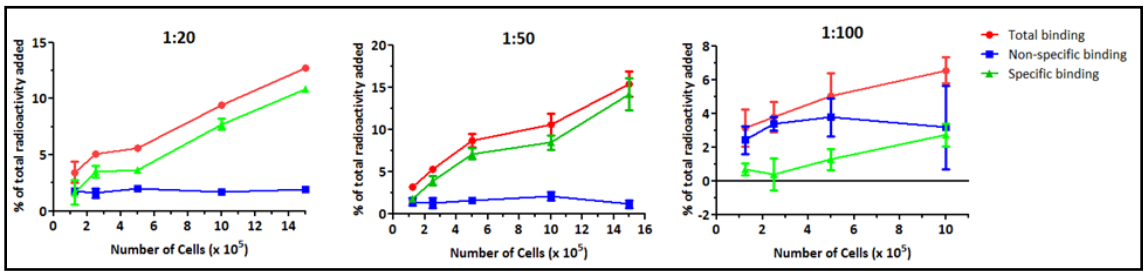

FIG. 7. Specific binding of ^{177}Lu-DOTA-Rituximab to Raji cell – conjugated prepared at different molar ratios (1:20, 1:50 and 1:100) (n = 3).

3.2.5 Conjugation of Anti-CD 20 antibody using 1B4M-DTPA chelating group and preliminary binding results

The antibody was conjugated with 1B4M-DTPA chelating group, gently provided by Dr. Zalutsky, at molar ratio 1:50. The purification after conjugation and labelling procedure were performed as described to DOTA-NHS chelating group. The result are expressed in the following table.

TABLE 8. Radiochemical purity of 1B4M-DTPA-Rituximab labelled with $^{177}LuCl_3$. Labelling conditions: 1 mg, 37 MBq (1 mCi) of $^{177}LuCl_3$, 1 hour at 43 °C, 350 rpm (n = 1).

Molar ratio	Before purification	After purification
1:50	32,99±0,007	92.56±0.001

Figure 8 shows the results of specific binding assay of ^{177}Lu-1B4M-DTPA-Rituximab to *Raji* cells.

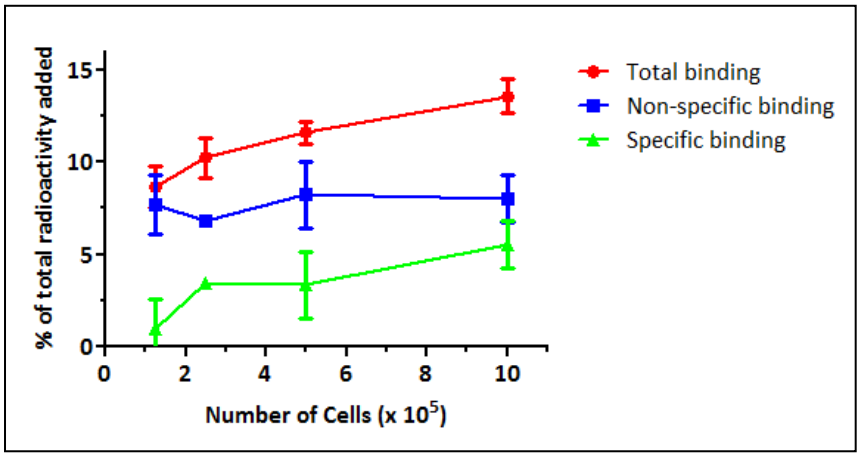

FIG. 8. Specific binding of ^{177}Lu-1B4M-Rituximab to Raji cell (n = 1).

3.2.6 Conjugation of Anti-CD 20 antibody using p-SCN-Bn-DOTA and p-SCN-Bn-DTPA

Preliminary conjugation and labelling studies were performed using p-SCN-Bn-DOTA and p-SCN-Bn-DTPA as chelators. The procedures for conjugation of the chelating group to the Ab and purification were the same employed for DOTA-NHS chelator, previously described. The labelling procedures were developed using 3.7 MBq (1.0 mCi) of ^{177}Lu and reaction conditions as previously described. The results are expressed on Table 9.

TABLE 9. Radiochemical purity of ^{177}Lu-DOTA-rituximab conjugated to two different chelators. Labelling conditions: 1 mg, 37 MBq (1 mCi) of $^{177}LuCl_3$, 1 hour at 43 °C, 350 rpm (n = 5).

	Radiochemical purity (%)	
Ab:chelator molar ratio	*p-SCN-Bn-DOTA.*	*p-SCN-Bn-DTPA.*
1:5	79.71 ± 1.40	-
1:10	88.04 ± 1.08	-
1:20	82.10 ± 0.32	76.68 ± 14.63
1:50	-	59.49 ± 9.10

3.3 Development of a radiopharmaceutical based on Bombesin for prostate and breast tumors radionuclide therapy

We have recently developed and described the properties of novel radiopharmaceuticals based on bombesin (BBN) structure and radiolabeled with Lutetium-177 – ^{177}Lu-DOTA-Phe-$(Gly)_{1-5}$-BBN(6-14) – for application in prostate tumor detection and treatment and we have obtained promising results. The derivative ^{177}Lu-DOTA-Phe-$(Gly)_5$-BBN(6-14) showed fast blood clearance ($T_{½}$ = 10 minutes), rapid renal excretion, low abdominal accumulation, short effective half-life and specifically target to human prostate tumor (PC-3) cells in mice [3]. We describe now, in this phase of the work, the toxicological aspects of this bombesin derivative in rats, in order to determine if it is safe for evaluation in clinical studies in humans. The toxicological studies analyze the adverse effects inherent to the administration of a pharmaceutical and are demanded by National Agency of Sanitary Surveillance (ANVISA) for radiopharmaceutical registration in Brazil.

Acute and chronic intravenous toxicity were evaluated by injecting 60 µg (150 µL of sterile saline) of the cold peptide DOTA-Phe-$(Gly)_5$-BBN(6-14) in rats (adults, male, 250 g) tail vein. The total mass injected was 10 times higher the mass that would be administrated to humans per kg, considering an adult of 70 kg, for radionuclide therapy. Rats' behavior was evaluated for two hours post injection and water and food intake as well as body weight were assessed daily. Moreover, after 24 hours and 7 days p.i., the animals were sacrificed in groups of five, the blood was collected for hematology and serum biochemistry and organs were dissected for histological evaluation.

Neither mortality nor changes in animals' behavior were observed during all times analyzed. Food and water intake, body weight, hematological (Fig. 28) and biochemical (Fig. 29 and 30) variables did not show differences of toxicological and/or statistical relevance between the experimental and control groups. In addition, microscopic examination of organs did not demonstrate any alterations and there were no histological findings of toxicological significance (Fig. 31). These results suggested that the bombesin derivative studied can be considered potentially safe for human use in clinical studies.

4. Future studies

4.1. Substance P

- Conclude *in vitro* cell binding assays with different tumor cells lines.
- Conclude biodistribution studies in *Nude* mice with tumor.
-

4.2. Anti CD-20 antibody (Rituximab)

- Determination of number of chelating groups per antibody using mass spectrometry;
- Prepare a lyophilized formulation for labelling the conjugated antibody with 177Lu – study of ideal formulation and stability of preparation;
- Conclude cell binding studies for the antibody conjugated with different chelators and study the influence of specific activity of the labelled antibody in tumor cell binding;
- Conclude biodistribution studies with the Ab prepared with different chelators and using different Ab:chelator molar ratios.

4.3. Bombesin

- Study new BBN derivatives: labelling conditions, biodistribution studies, cell binding assays – comparative study with the BBN derivative previously studied.

5. Published works during the related period

Full length articles

Pujatti, P.B., J.S. Santos, R.M. Couto, L.T. Melero, M.F. Suzuki, C.R. Soares, S.R. Grallert, J. Mengatti, and E.B. De Araujo. Novel series of (^{177})Lu-labeled bombesin derivatives with amino acidic spacers for selective targeting of human PC-3 prostate tumor cells. *Q J Nucl Med Mol Imaging*. 55:310-323, 2011.

Abstracts in events

Priscilla B. Pujatti, Jair Mengatti, Elaine Bortoleti de Araújo, Comparision of in vitro serum stability and in vivo properties of spacer-modified-bombesin conjugates with methionine and norleucine as C-terminal amino acids. 24th Annual Congress of the European association of Nuclear Medicine – EANM'11, October 15-19, 2011, Birmingham, UK. Eur. J. Nucl. Med. Mol. Imaging 38 (Suppl 2): S93-S228, OP378.

Priscilla B. Pujatti, Nathanael Gomes, Akin G. Akanji, Jair Mengatti, Elaine Bortoleti de Araújo. Preclinical evaluation of a radiopharmaceutical for refractory prostate tumor radionuclide therapy. Alasbimn Journal Year 14, Number 54, October 2011 / Año 14, Nº 54, Octubre 2011, XXIII Congreso de ALASBIMN: Resúmenes.

Clarice M. de Lima, Priscilla B. Pujatti, Maria T. Colturato, Jair Mengatti, Elaine Bortoleti de Araújo Evaluation of methionine oxidation on ^{177}Lu-DOTA-Substance P on in viro binding to glioma cells. Alasbimn Journal Year 14, Number 54, October 2011 / Año 14, Nº 54, Octubre 2011, XXIII Congreso de ALASBIMN: Resúmenes.

Adriana V. F. Massicano, Priscila B. Pujatti, Jair Mengatti, Elaine Bortoleti de Araújo Mass influence of DOTA-Rituximab in the radiolabelling with Lu-177. Alasbimn Journal Year 14, Number 54, October 2011 / Año 14, Nº 54, Octubre 2011, XXIII Congreso de ALASBIMN: Resúmenes.

Massicano, A.V.F.; Alcarde, L.F.; Mengatti, J.; Araújo, E.B. Labelling of Rituximab with 177Lu: specific cell binding assays. Brazilian Congress of Nuclear Medicine, Salvador, Bahia, October, 10-14, 2012 – Accepted for poster presentation.

Oliveira, R.S; Pujatti, P.B; Massicano, A.V.F.; Mengatti, J.; Araújo, E.B. Determination of standard condition for labelling Bombesin derivative with ^{111}In. Brazilian Congress of Nuclear Medicine, Salvador, Bahia, October, 10-14, 2012 – Accepted for poster presentation.

Priscilla B. Pujatti; Jane K. Sosabowski; Julie M. Foster; Ciara Finucane; Chantelle D. Hudson; Jerome C. Burnet; Jair Mengatti; Stephen J. Mather, and Elaine B. de Araújo. Comparative biodistribution and nanoSPECT/CT imaging of a new [In-111]-DOTA-bombesin derivative in low and high GRPr expressing prostate tumor cells. Brazilian Congress of Nuclear Medicine, Salvador, Bahia, October, 10-14, 2012 – Accepted for oral presentation.

6. Post graduation students participating in this work

Priscilla B Pujatti – PhD – concluded in 2012
Guilherme Luiz Carvalho – PhD
Adriana Massicano – PhD
Akinkumi Akanji – PhD
Renata Martinussi Couto – PhD
Ricardo Oliveira - MSc

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CHINA

Development of Therapeutic Radiopharmaceuticals Based on ^{177}Lu Labelled Monoclonal Antibodies

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1. Introduction

The overall objective of this CRP is to promote the clinical application of radioimmunotherapy and peptide receptor-based radionuclide therapy for the most important cancers in developing countries.

This report summarized the ongoing activities of our group within the first half of this CRP.

2. Production of ^{177}Lu

Smaller quartz glass tubes (ϕ, 5 × 5 mm) were used to improve the discovery rate of ^{177}Lu in the chemical process. 1mg of $^{176}Lu_2O_3$ (82.8% enriched) was dissolved in 6 mol/L HCl and 30% H_2O_2 (V/V = 1:1) and the clear solution was dispersed in quartz glass tubes (ϕ, 5 × 5 mm) and then evaporated to dryness. After irradiated for one cycle (9-11 days) under the neutron flux of $2\text{-}4\times10^{13}$, the target was directly dissolved in 0.2 mol/L HCl. The radionuclidic purity in all circumstances was over 99.9%.

Lu content was determined using ARS III spectrophotometry. Briefly, standard Lu solution, sample solution, 1.0 mL 0.5% ARS III and 0.5mL 1mol/L HCl were mixed together, and after 5 min of color reaction, the absorbance was measured at 655 nm. The obtained specific activity of ^{177}Lu was 2.5-2.9 Ci/mg.

TABLE 1. The production of ^{177}Lu (some examples)

EOB (date)	$^{176}Lu_2O_3$ (μg)	Chemical processing (date)	^{177}Lu(mCi)[a]	^{177}Lu (mCi/mL)[b]	Yield (%)[c]	S.A. (mCi/mgLu) [d]
20111013	20	20111020	29	15/0.3mL	51.7	2900/1450
20120314	60	20120316	151	141/0.9mL	93.4	2517/2275
20120412	60	20120413	170	147/0.9mL	86.5	2833/2577
20120517	60	20120518	174	150/0.55mL	86.2	2900/2639
20120712	60	20120713	127	110/0.5mL	86.6	2117/1926
20120816	60	20120817	174	143/0.55mL	82.2	2900/2640

[a] Radioactivity before chemical process. [b] Radioactivity after chemical process. [c] Recovery rate. [d] Specific activity after chemical process / specific activity when labelling started.

3. Conjugation of Nimotuzumab with CHX-A''-DTPA and p-SCN-Bn-DOTA

3.1. Purification of Nimotuzumab

1.0 mL of Nimotuzumab (5 mg/mL) was purified with ultrafiltration centrifugal tube (Vivaspin 2, MWCO 30,000, Sartorius). After incubated with 0.5 mL of 40 mM DTPA (in 0.25 M sodium acetate buffer, pH = 6.5-7.0) at 4 °C for 30 min, the solution was centrifuged at 4000rpm/min and washed with 6 × 1mL of 0.1 M carbonate buffer (pH = 8.3-9.0). After purification, the concentration of antibody was determined using UV spectrophotometry at 280 nm, and the recovery rate was around 70%.

3.2 Conjugation of Nimotuzumab with chelators

3.2.1. Conjugation of Nimotuzumab with CHX-A''-DTPA

Purified Nimotuzumab was mixed with 10～250 folds molar excess of CHX-A''-DTPA (15 mg/mL) in 50 mM bicarbonate buffer (pH = 8.3-9.0). After incubated at 4 °C for 17h, the reaction mixture was transferred in Vivaspin 2 tube and centrifuged at 4000 rpm/min, then 10 × 0.5 mL acetate buffer (20 mM, pH = 6.5-7.0) was added to change medium. The conjugate solution was stored at 4°C.

3.2.2. Conjugation of Nimotuzumab with p-SCN-Bn-DOTA

Purified Nimotuzumab was mixed with 30～350 folds molar excess of p-SCN-Bn-DOTA (1mg/mL) in 50 mM bicarbonate buffer (pH = 8.3-9.0). After incubated at 4 °C for 42 h, the reaction mixture was transferred in Vivaspin 2 tube and centrifuged at 4000 rpm/min, then 10 × 0.5ml acetate buffer (20 mM, pH = 6.5~7.0) was added to change medium. The conjugate solution was stored at 4°C.

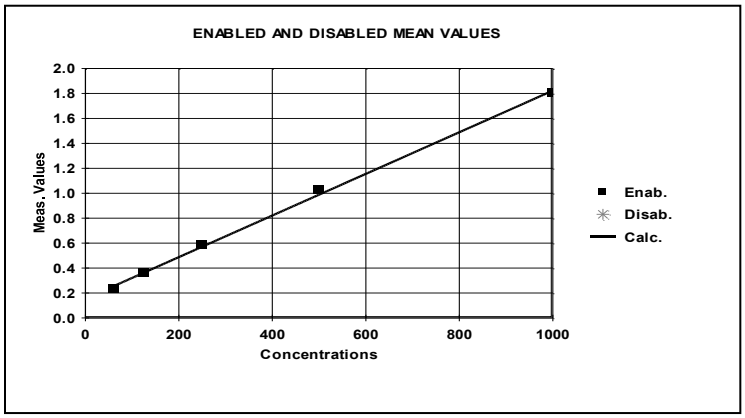
3.3. Measurement of chelator to antibody ratio

3.3.1. Measurement of the concentration of antibody in mAb conjugate

The concentration of antibody was measured by BCA protein assay kit.

Standard antibody solutions were made by dissolving purified Nimotuzumab in sodium acetate buffer (20 mM, pH = 6.5-7.0), and final concertration of antibody was 1000, 500, 250, 125, 62.5 μg/mL.

Each of above antibody solution was tested by microplate reader (MK3, Thermo-Fisher SCIENTIFIC). Briefly, 25 μL of standard antibody solutions were mixed with 200 μL of BCA solution in 96-well plate (Brand plates, immunograde) and tested at 570 nm after 2 h of color reaction, and acetate buffer was used as reference solution. The standard concentration- absorbance curve was shown in Figure 1.



Fit type:	Linear regression (SVD): y = a+b*x	
Parameters:	a	b
	0.154	0.002
Corr. coeff. R2:	0.996	

FIG. 1. The standard curve for measurement of antibody in mAb conjugates.

Then 25 μL of conjugate sample was tested in the same way described above, and the concentration of Nimotuzumab in mAb conjugate was about 2.5437 mg/mL, 1.696×10^{-5}M according to the standard curve.

3.3.2. Measurement of the concentration of DTPA in mAb conjugate

The density of DTPA in mAb conjugate was determined according to the literature method [1].

3.3.2.1. Preparation of Y-ARSIII solution

Arsenazo III (ARS III) 4.2 mg and $YCl_3 \cdot 6H_2O$ 6.7 mg were dissoveled in 100 mL acetate buffer (0.2 M, pH = 4), respectively. 10mL of ARS III solution and 1mL of YCl_3 solution were mixed in 100 mL acetate buffer to get Y-ARSIII color solution.

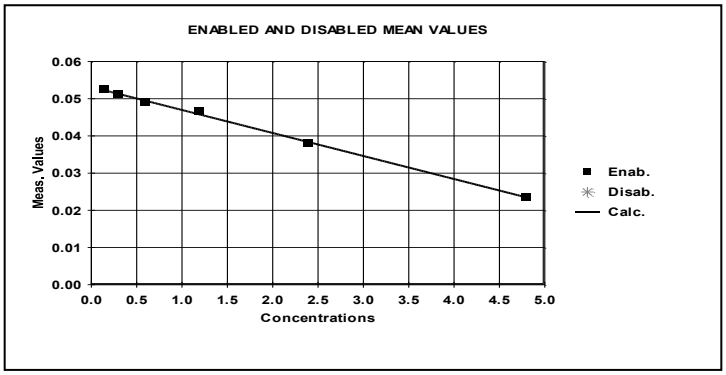
3.3.2.2. Preparation of standard cuver

Standard DTPA chelator solutions were made by dissolving 4.8 mg of CHX-A"-DTPA in 100 mL acetate buffer(0.2 M, pH = 4), and final concertration of chelator was 48, 24, 12, 6, 3, 1.5 μg/mL.

30 μL of standard chelator solutions were mixed with 270 μL of Y-ARSIII solution and tested at 650nm after 5 min of color reaction, and acetate buffer (pH = 4, 0.2 M) was used as reference solution. The standard concentration- absorbance curve was shown in Figure 2.

30 μL of conjugate sample was tested, and the concentration of DTPA in the conjugate was about 10.92 μg/mL, 1.838×10^{-5}M according to the standard curve.

Therefore, the ratio of CHX-A"-DTPA to mAb was 1.08:1.



Fit type:	Linear regression (SVD): y = a+b*x	
Parameters:	a	b
	0.053	-0.006

Corr. coeff. R2:	0.997

FIG. 2. The standard curve for measurement of DTPA in mAb conjugates.

3.3.3. Measurement of the concentration of DOTA in mAb conjugate

The density of DOTA in mAb conjugate was determined according to the literature method [2].

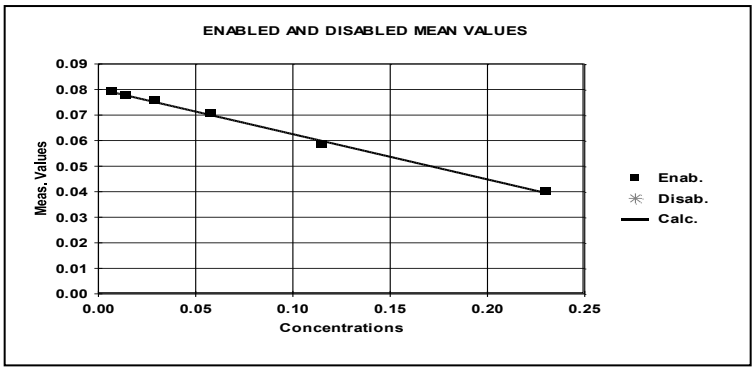
3.3.3.1. Preparation of ARS-Cu solution

Arsenazo III (ARS III) 0.16 mg was dissoveled in 20 mL ammonium acetate buffer (0.2M, pH = 4) and mixed with 6.4 μL Cu standard solution (Cu: 1 mg/mL) to get stock solution.

3.3.3.2. Preparation of standard cuver

Standard DOTA chelator solutions were made by dissolving p-SCN-Bn-DOTA 0.46 mg in 2 mL ammonium acetate buffer (0.2M, pH = 6.5), and final concertration of chelator was 230, 115, 57.5, 28.8, 14.4, 7.2 μg/mL.

20 μL of standard chelator solutions were mixed with 180 μL of stock solution and tested at 630 nm after 20 min of color reaction, and acetate buffer was used as reference solution. The standard concentration-absorbance curve was shown in Figure 3.



Fit type:	Linear regression (SVD): y = a+b*x	
Parameters:	a	b
	0.080	-0.177
Corr. coeff. R2:	0.995	

FIG. 3. The standard curve for measurement of DOTA in mAb conjugates.

3.4. Discussion

1) Conjugation should be carried out under alkaline condition (bicarbonate buffer (pH = 8.3-9.0)). There seemed to be no coupling under neutral condition in our experiments.
2) It takes longer for DOTA conjugation than for DTPA conjugation.

3) Measurement of DOTA concentration in the conjugate using spectrophotometry was not successful. We will try the method of radiolabelling [6].
4) Though the inlets of chelator to mAb were quite different, but the ratio of chelator/mAb of the conjugate didn't show much difference (see Table 2).

4. ^{177}Lu labeling of mAb conjugates

4.1. Preparation and purification of ^{177}Lu mAb conjugates

Typical labelling procedure: Mab conjugate(200ug) was mixed with $^{177}LuCl_3$ (2.0 mCi/10μL) in sodium acetate buffer (pH = 6.5-7.0). After vortex, the mixture was incubated at 37°C (for DTPA conjugates) or at 40°C (mainly for DOTA conjugates) with shaking speed of 650rpm for 60min then the reaction was quenched by adding 50 μL of 10 mM DTPA. The results are shown in Table 2-5.

TABLE 2. ^{177}Lu labelling of DTPA-Nimotuzumab

DTPA/mAb inlet	DTPA/mAb detected	Lu/mAb	pH	RCP (%)
200:1	1.08:1	3:1	7	52.7
200:1	1.08:1	1.4:1	7	57.7
144:1	--	10:1	7	99.6
144:1	--	0.3:1	7	98.6
55.5:1	--	2:1	7	97.3
55.5:1	--	1:1	7	92.3
55.5:1	--	0.5:1	7	91.7
50:1	--	3:1	7	21.6
50:1	--	1.4:1	7	70.6
12.5:1	1.07:1	3:1	7	32.2
12.5:1	1.07:1	1.4:1	7	90.2

TABLE 3. ^{177}Lu labelling of DTPA-Rituximab

DTPA/mAb	Lu/mAb	pH	RCP (%)
240:1	2:1	7	69.8
240:1	1:1	7	66.1
240:1	0.5:1	7	58.5

TABLE 4. ^{177}Lu labelling of DOTA-Nimotuzumab

DOTA/mAb	Lu/mAb	pH	RCP (%)

340:1	3:1	7.5	84.9
340:1	3:1	5.5	96.5
340:1	3:1	4.8	98.4
200:1	5:1	7	67.8
50:1	2.4:1	7	99.4
50:1	1.5:1	7	99.0
46:1	2:1	7	95.2
46:1	1.1: 1	7	97.7

TABLE 5. ^{177}Lu labelling of DOTA-Rituximab

DOTA/mAb	Lu/mAb	pH	RCP (%)
30:1	2.3:1	7	96.9
30:1	1.1:1	7	96.9
30:1	0.5:1	7	98.7

The labelled mAb was purified using PD-10 column eluted with PBS, and the typical eluting curve was shown in Figure 4.

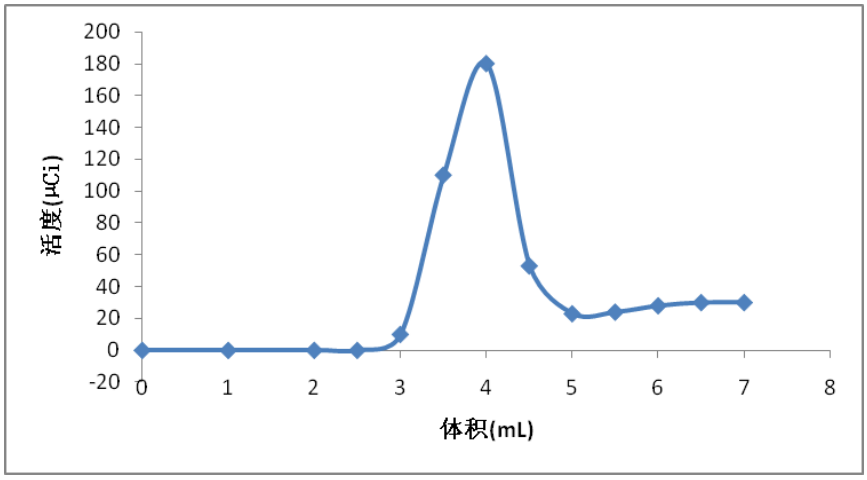


FIG. 4. The eluting curve of purification of labelled mAb conjugates with PD-10

Lu-177 labelled mAb conjugates were mainly in 3.0 mL~5.0 mLof elution solution. The labeling yield was over 90%, and RCP could reach 99% after purified with PD-10 column.

This conjugated mAb was, and the concentration of mAb was 2.37 mg/ml as we checked before labelling. But mainly because of the low S.A of ^{177}Lu, which was 1.5 Ci/mg when we did the labelling, the labelling yield was quite low.

The labelled mAb was purified using PD-10 column (pre-treated with 0.5% human serum albumin) eluted with saline.

4.2. Influence of pH and reation time

DOTA-Nimotuzumab (180ug) was mixed with $^{177}LuCl_3$ (1.5 mCi/6μL) in sodium acetate buffer, and pH was adjusted to 7.5, 5.5, 4.7 respectively with 36% acetic acid. The results are showed in Table 6.

TABLE 6. The influence of pH to the labelling reaction

pH	7.76	5.25	4.53
Labelling yield (%)	84.9	96.5	98.4

During labelling, the samples were taken for RCP analysis, and the results showed that from 30 min to 60 min, the RCP increased from 85.2% to 90.2%.

4.3. Discussion

1) Though most of labelling experiments were done in neutral condition, acid condition seemed to be more favorable.
2) Labelling yield could be increased when reaction time was prolonged or Lu/mAb ratio was increased.
3) During PD-10 purification, labelled conjugate mainly in eluate 3-5mL, but 3-4mL seemed to be purer.

5. Analysis of RCP

5.1. ITLC analysis

Using ITLC-SG as support, the samples were analysized in following different developing solvents, and based on the results, EtOH:NH_4OH:H_2O(2:1:5) and 10mM DTPA were chosen to be main developing solvents.

TABLE 7. The R_f in differant systems

Conditions	R_f		
	^{177}Lu-mAb conjugate	^{177}Lu-chelator	Free ^{177}Lu
10%NH_4OAc:CH_3OH (1:1)	0.0-0.2	0.8-1.0	0.0-0.5
EtOH:NH_4OH:H_2O (2:1:5)	0.0-0.2	0.8-1.0	0.0-0.2
14%NaOAc	0.0-0.2	0.3-0.5	0.0-0.2
0.1M Sodium citrate	0.0-0.2	--	0.9-1.0
10mM DTPA	0.0-0.1	0.9-1.0	0.9-1.0
Acetone:saline = 1:1	0.0-0.2	0.0-0.2	0.0-0.2

5.2. HPLC analysis

HPLC was performed on size exclusion column using 0.9% NaCl/0.05%NaN_3 as eluting solvent. The results were shown in Figure 5.

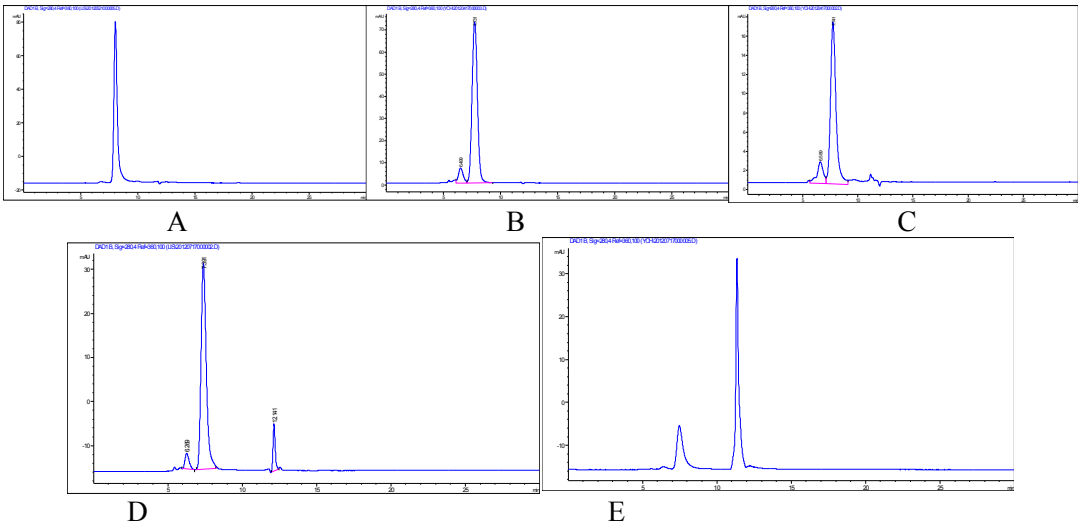
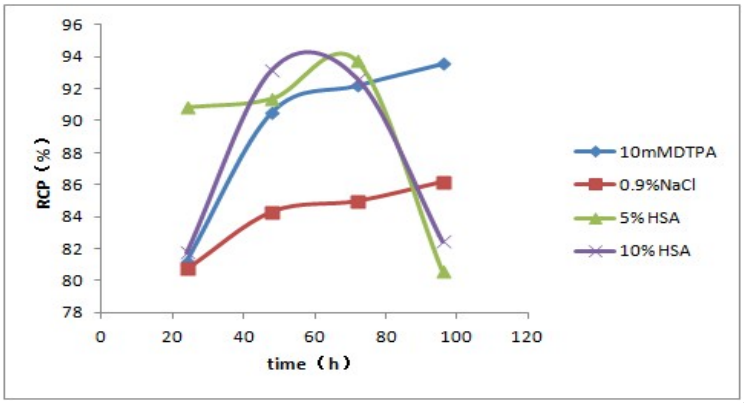


FIG. 5. HPLC profoles. A: Nimotuzumab, Rt = 8.05min; B: DTPA-Nimotuzumab, Rt = 7.73min; C: ^{177}Lu-DTPA-Nimotuzumab, Rt = 7.74min; D: DOTA-Nimotuzumab, Rt = 7.39min; E: ^{177}Lu-DOTA- Nimotuzumab, Rt = 7.48min

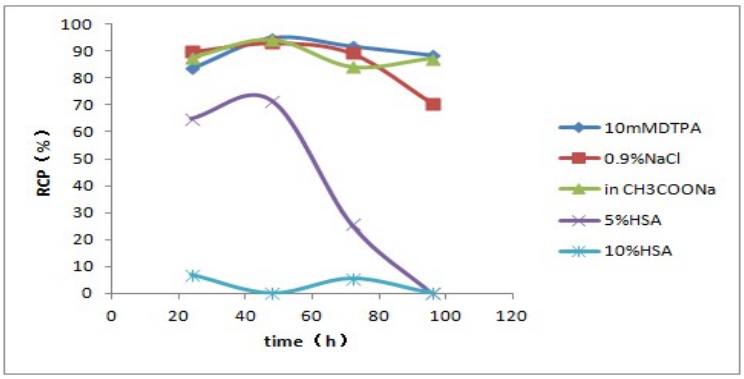
6. In-vitro stability

Lu-177 labelled Nimotuzumab conjugates were incubated in 0.9%NaCl, 10mM DTPA, 5%HSA and 10%HSA at 37°C, and samples were taken at 24h, 48h, 72h and 96h for ITLC analysis. The results were shown in Figure 6.

The results showed that ^{177}Lu labelled mAb conjugates were relatively stable in 0.9% NaCl and 10-mM DTPA, but not stable in 5%HSA and 10%HSA.



A



B

FIG. 6. In-vitro stability. A: ^{177}Lu-DTPA-Nimotuzumab; B: ^{177}Lu-DOTA- Nimotuzumab.

7. Biodistribution study in normal mice

A typical procedure was as follows. normal Kunming mice (18-22g) were injected with 0.37 MBq/0.1 mL of ^{177}Lu labelled Nimotuzumab conjugates via tail vein. Animals were killed at 24hr and 48hr p.i. and samples of different organs were dissected and counted. The uptake in terms of %ID/g was calculated. Table 8 and Figure 7 showed the biodistribution of ^{177}Lu-DTPA-Nimotuzumab in Kunming mice (N=5). Table 9 and Figure 8 showed the biodistribution of ^{177}Lu-DOTA-Nimotuzumab in Kunming mice (N=5).

TABLE 8. The biodistribution of ^{177}Lu-DTPA-Nimotuzumab in Kunming mice (N = 5)

Organs	24 h p.i.	48 h p.i.
Heart	1.84±0.58%	2.11±0.35%
Liver	5.58±1.33%	4.79±0.64%
Spleen	3.71±1.16%	4.50±0.91%
Lung	2.78±0.60%	2.43±0.38%
Kidney	9.23±1.84%	9.32±0.77%
Stomach	1.07±0.52%	1.34±0.74%
Small Intestine	0.96±0.18%	0.98±0.07%
Muscle	0.58±0.09%	0.82±0.09%
Bone	3.68±1.04%	4.47±0.43%
Blood	5.61±1.18%	4.64±0.30%

TABLE 9. The biodistribution of ^{177}Lu-DOTA-Nimotuzumab in Kunming mice (N = 5)

Organs	24 h p.i.	48 h p.i.
Heart	1.42±0.26%	0.39±0.20%
Liver	14.44±1.47%	3.85±0.64%
Spleen	11.06±2.16%	3.58±0.46%

Lung	1.19±0.24%	0.36±0.2%
Kidney	10.78±1.27%	2.96±0.24%
Stomach	0.72±0.15%	0.23±0.12%
Small Intestine	0.78±0.16%	0.27±0.16%
Muscle	0.50±0.13%	0.07±0.06%
Bone	4.73±0.53%	1.91±0.40%
Blood	1.45±0.18%	0.31±0.10%

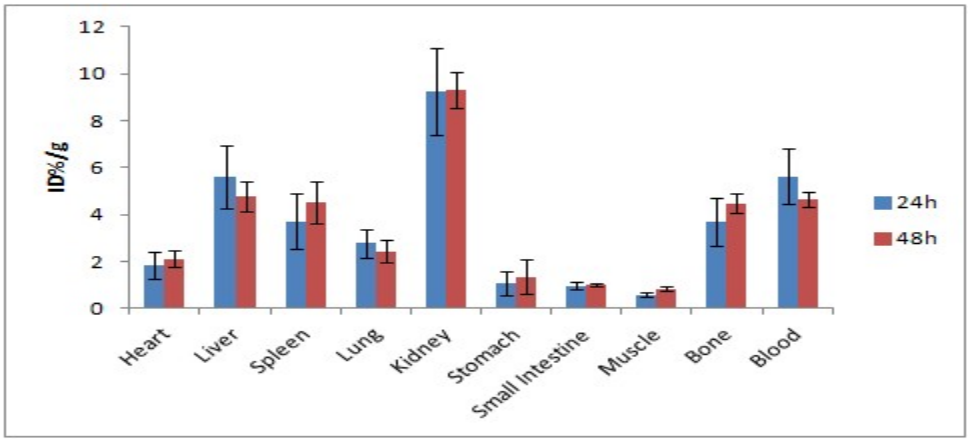


FIG. 7. The biodistribution of ^{177}Lu-DTPA-Nimotuzumab in Kunming mice

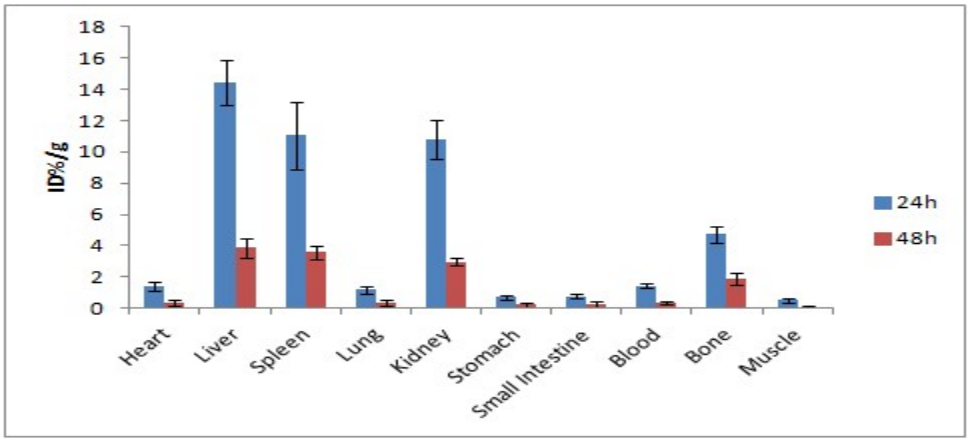


FIG.8. The biodistribution of ^{177}Lu-DTPA-Nimotuzumab in Kunming mice

Rapid blood clearance and high kidney uptake showed that ^{177}Lu-DOTA-Nimotuzumab may be degraded in-vivo which was consistent with its in-vitro instability. ^{177}Lu-DTPA-Nimotuzumab also showed high kidney uptake besides high uptake in liver and blood. Further data from HPLC was needed to confirm the results from stability studies.

8. Future work plan

1) Radioimmunoassay of Nimotuzumab will be performed using LoVo colorectal cells.
2) More experiments on Rituximab and new chelators will be carried out.

ACKNOWLEDGEMENTS

The authors are grateful to IAEA for all the supports and helps.

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CUBA

Preclinical evaluation of ^{90}Y labeled biomolecules (Rituximab, hR3, Substance-P) as potential therapeutic radiopharmaceuticals

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1. Description of research work

1. Experiments and adjustment of labelling procedures for Rituximab with ^{90}Y.
2. Determination of the conditions under which DOTA and DTPA immunoconjugates can be labeled with ^{90}Y in high yield.
3. Quality Controls and binding assay of conjugated antibodies.

2. Results

The main contribution of our group in this period was focused in conditions for conjugation of rituximab with DTPA and DOTA chelators.

2.1. DOTA conjugation

Rituximab (15mg/mL) was incubated overnight at room temperature with HHS-DOTA in 0.1M PBS pH 8.5 at different molar ratios (160:1, 80:1, and 40:1). Then, a purification step by exclusion chromatography in Sephadex column (PD10, Pharmacia) was performed.

2.2. DTPA conjugation

Rituximab (10 mg/mL) was incubated overnight at room temperature with CHX-DTPA in NaHCO3 Buffer 0.1 M, pH 8.5, at different molar ratios (30:1, 20:1, 10:1, and 5:1). Then, a purification step by exclusion chromatography in Sephadex column (PD10, Pharmacia) was performed.

Conjugated antibodies were concentrated by mean of spin concentration and fractions of 10mg of conjugated antibody were stored in a fridge in 1mL eppendorff test vials until use.

2.3. Binding experiments of conjugated antibodies to Ramos cells

2×10^5 cells were incubated for 30 min with 10 μg/mL of conjugated antibody. Then, cells were washed with physiological saline and centrifuged at 2000 rpm for 2 min. After a second incubation step with a rabbit anti human IgG (Fcγ) coupled to FITC (*fluorescein iso-thiocyanate,* Dako, Denmark) in ice for 30 min, a flow cytometry analysis was performed (FACScan, Becton Dickinson, USA).

Binding capacity was evaluated at different Ab/DOTA molar ratios. As can be observed in Figure 1, binding of conjugated antibody decreases with respect to free Rituximab when molar ratio increases, which is more evident at 80:1 and 160:1 molar ratios.

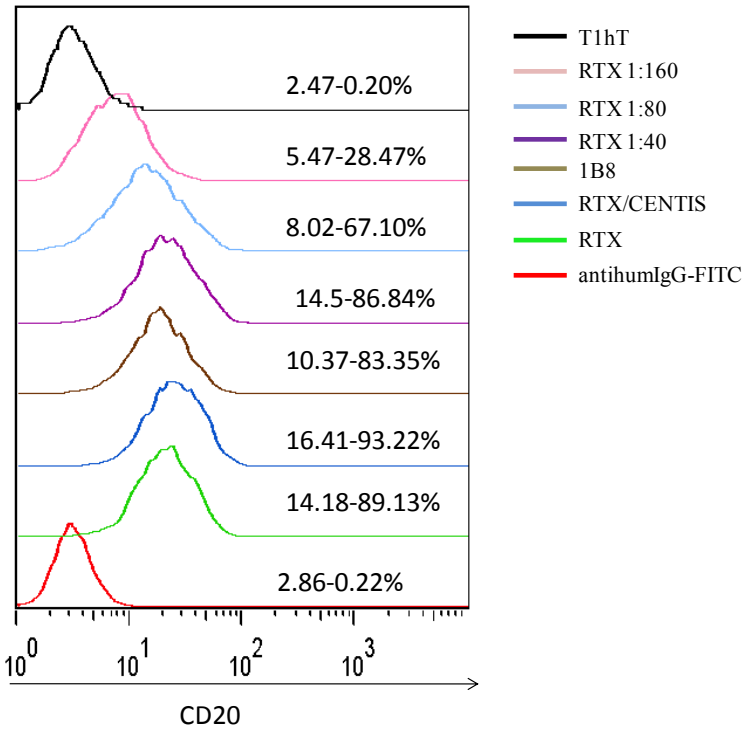

FIG. 1. CD20 recognition in Ramos cells of DOTA conjugated Rituxan (numbers represent fluorescence intensity and percentage of CD20 expressing cells).

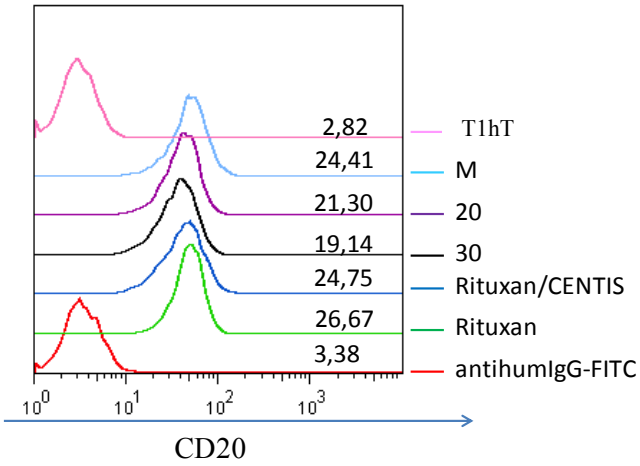

FIG. 2. CD20 recognition in Ramos cells of DTPA conjugated Rituxan (numbers represent fluorescence intensity).

FITC conjugated antibody, is Fcγ specific. So, a binding decreasement can be due to poor CD20 recognition or Fc fragment damage. Nonetheless, both factors are very important in "in vivo" behavior of radiolabeled antibodies.

2.4. ^{90}Y radiolabeling and quality control of conjugated Rituximab

In the case of DOTA conjugated Rituximab, radiolabeling was carried out at 42°C, acetate buffer 0.25 M, pH 7.0, for 90 minutes.

In the case of DTPA conjugated Rituximab, radiolabeling was carried out at room temperature in acetate buffer 0.25 M, pH 5.5, during 15 minutes.

Quality control of radiolabeling was performed by paper chromatography and ITLC.

TABLE 1. Chromatographic method for quality control of radiolabeled Abs

System	Mobile phase
Paper, Whatman 3MM	NH4Ac 0.1 M pH 5.5-6.0, EDTA 50 mM
ITLC	MeOH:NH3 10% (1:1)

TABLE 2. Radiochemical purity of radiolabeled MAbs

DOTA-Ab		DTPA-Ab	
Molar ratio	RP	Molar ratio	RP
160:1	97-99 %	30:1	96-98 %
80:1	90-95 %	20:1	96-98 %
40:1	~ 80 %	10:1	96-98 %
		5:1	10-12 %

Final radiochemical purity of DTPA conjugated antibody was initially superior to radiochemical purity obtained with DOTA conjugated antibody. Better results, more than 90% of radiochemical purity, were further obtained with DOTA conjugated at lower molar ratio (20:1).

2.5. Cell binding assay of ^{90}Y-DTPA-Rituximab

Ramos cell line (CRL-1596, ATCC) was used for binding experiments. Cells were grown, 37ºC in 5% CO_2, in RPMI 1640 (Gibco, UK) media supplemented with penicilline (100 U/mL), streptomicine (100 µg/mL) and fetal calf serum 10%. For binding experiments cells were centrifuged and washed in isotonic PBS/BSA pH 7.4 and resuspended in the same buffer at different concentrations (2.4, 1.2, 0.6, 0.3, 0.15 and 0.075 x10^6 cell/mL). The conjugate DTPA-Rituximab obtained at 20:1 molar ratio was used for cell binding experiments after labeling.

By duplicate, 10µL of a conveniently diluted radiolabeled antibody (5000cpm) was added to 1mL of each cell dilution.

Cell binding was processed following the variable antigen concentration approach according to already described procedures (Konishi, et al, 2004). Conventional and Lindmo plot (Lindmo et al, 1984) are depicted in Figure 3.

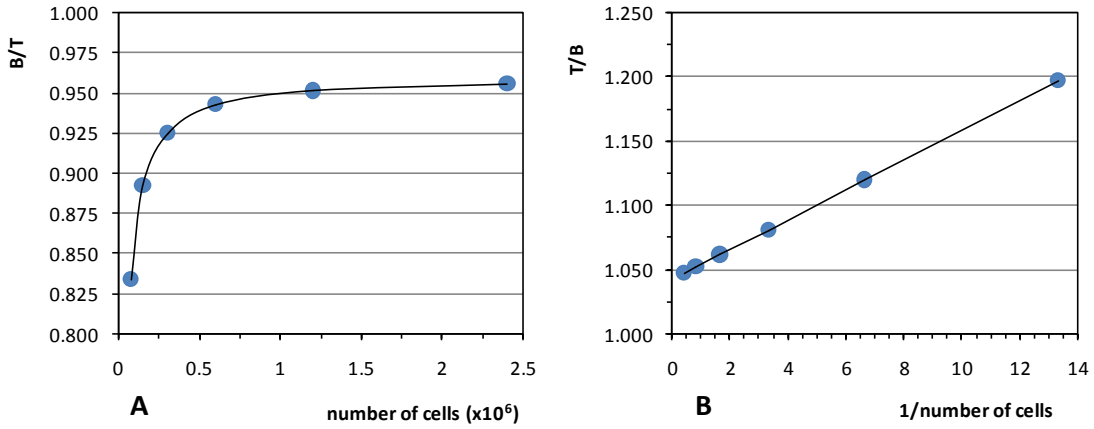

FIG. 3. Results of binding experiment. A: conventional plot, B: Lidmo plot.

The value of immunoreactive fraction (r) of 0.96, as estimated by both procedures, is an excellent result. So, we may consider that conjugation procedures did not affect binding capacity of Rituximab.

3. Conclusions

1. Rituximab conjugated with DOTA and DTPA shows a good binding capacity to Ramos cells in vitro. DOTA-rituximab at 40:1 molar ratio and DTPA-Rituximab at 20:1 molar ratio as demonstrated by flow cytometry.
2. ^{90}Y labeled DOTA-Rituximab and DTPA-Rituximab were obtained with more than 90% of radiocemical purity.
3. ^{90}Y labeled DTPA-Rituximab showed a good receptor recognition in Ramos cells as demonstrated by binding experiments.

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CZECH REPUBLIC

Therapeutic radiopharmaceuticals based on ^{177}Lu-and ^{90}Y-labelled monoclonal antibodies and peptides: development and preclinical evaluations

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1. Introduction

The investigation of target-specific radiopharmaceuticals based on monoclonal antibodies (mAb), their fragments and peptides for therapy and molecular imaging is increasing due to the availability of new radioisotopes and biomolecules with improved characteristics. Unfortunately, the success of radioimmunotherapy (RIT) in hematologic disease has not been translated to solid tumours yet. Nevertheless, treatment of minimal residual disease, locoregional applications and pretargeted RIT has shown some advances [1].

The epidermal growth factor receptor (EGFR) is a rational target of anticancer therapies due to their overexpression in a variety of malignant epithelial tumours and its correlation with a poor prognosis[2]. One of the approaches most widely studied to inhibit the EGFR oncogenic expression is the use of monoclonal antibodies which bind the extracellular domain of this receptor inhibiting dimerization and autophosporylation [3]. Trastuzumab was the first mAb approved by FDA for the treatment of HER-2 positive breast cancer. Later, cetuximab (Erbitux) was approved for treatment of metastatic colorectal cancer and squamous cell carcinoma. Relatively news mAbs against EGFRs such as Panitumumab, Matuzumab, MDX-447, Nimotuzumab and mAb806 are currently under preclinical and clinical evaluation [2, 4]. Tc-99m-h-R3 has been used for radioimmunoscintigraphy of nodal metastatic disease, but poor correlation between EGFR expression and positive tumour imaging was observed [5]. However, encouraging results have been obtained in RIT (phase I clinical trial) of recurrent high-grade glioma after intracavitary administration of h-R3 labelled with ^{188}Re [6].
Residualizing radionuclides, such as ^{90}Y and ^{177}Lu, are potentially more suitable for RIT [7]. Y-90 is of particular interest for radiotherapy due to its high-energy pure β-particle emission[8]. ^{90}Y form very stable complex with 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate acid (DOTA) derivatives *in vivo*, hence it is well suited for receptor-based therapeutic radiopharmaceuticals ^{177}Lu ($t_{1/2}$ = 6.7 days, Eγ = 0.208 MeV, β^-= 0.497 MeV, max range in tissue penetration = 2.0 mm) is being strongly considered for RIT since it combines the advantages of both ^{90}Y and ^{131}I. In addition, ^{177}Lu emits two low energy γ lines with energy of 113 and 208 keV that are suitable for imaging and assessment of delivered doses.

Hereby, we describe the radiochemistry and preclinical evaluation for the successful preparation of radioimmunoconjugates formed by ^{90}Y/^{177}Lu and monoclonal antibodies with intermediate affinity to EGFR (Nimotuzumab) and HER2/neu (Trastuzumab). In addition, Rituximab was modified with p-SCN-Bn-DOTA and 1B4M-DTPA and radiolabeled with ^{90}Y. Part of the following results has been published [7-11].

2. Materials and methods

2.1. Conjugation of monoclonal antibodies

Modification of Nimotuzumab and Trastuzumab with DOTA-NHS, p-SCN-Bn-DOTA and p-SCN-Bn-DTPA (Macrocyclics (Dallas, TX, USA) were performed as previously described [9-11]. Monoclonal antibodies were mixed with 10, 20, 50,100 and 150-fold molar excess of the selected bifunctional chelating agent. Rituximab was

modified with 20-fold molar excess of p-SCN-Bn-DOTA and 1B4MDTPA. The reaction mixture was incubated overnight at 4 °C. The immunoconjugates were purified by ultrafiltration on Vivaspin 6 until the absorbance in the ultrafiltrate at 280 nm was nearly zero. Protein concentration was determined by Bradford assay. Conjugates were stored at 4°C for further use.

2.2. Characterization

Immunoconjugates were characterized by SE-HPLC to verify conjugate integrity. About 20 µL of conjugates was injected onto a TSK-Gel SW 3000 (7.5× 300 mm, 10 µm, TosoHass) column using NaCl (0.9%)/NaN_3 (0.05%) as the mobile phase. The flow rate was maintained at 1 mL/min and the elution was monitored by UV at 280 nm. Immunoconjugates were also analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) under reducing and nonreducing conditions using NuPAGE® minigels 4-12 % Bis-Tris (10 mm × 10 well). The electrophoresis running was carried out in an Amershan Biosciences electrophoretical systems using NuPAGE® running buffer. The proteins in the gel were visualized with Coomassie Blue R-250 (BioRad, CA, USA) solutions.

2.3. Determination of average number of chelates linked to an antibody molecule

The average number of chelates linked to an antibody molecule was determined using ^{90}Y by a radioactive method previously described [12]. Briefly, 49 µg (10 µL) of the conjugates were added to 30-50 µL of NH4OAc 0.5 mol/L, pH 7.0. Afterward, 10 -30 µL (3.33 X 10^{-4} mol/L) of a standardized YCl_3 spiked with ^{90}Y solution were added.

2.4. F(ab')2 antibody fragments generation

Monoclonal antibodies were digested with 6.5% (w/w) of immobilized pepsin for 4 – 48 hours at 37ºC in 0.02M sodium acetate buffer, pH 4.1. The F(ab')2 fragments were purified by protein A column chromatography followed by elution with PBS 0.05M pH – 7.1. The F(ab')2 where concentrated by ultracentrifugation using Vivaspin 6 (MWCO = 30 kDa) as described above. The digestion and purity of F(ab')2 preparation was evaluated by SDS-PAGE under nonreducing conditions and SE-HPLC. The HPLC procedure was carried out using TSK-Gel SW 3000 and Bio Rad SEC 250 columns in series to increase the resolution. The F(ab')2 were modified with p-SCN-Bn-DOTA using the same procedure as described above for the intact monoclonal antibodies.

2.5. Radiolabeling

Lu-177 no-carrier added (n.c.a in 0.05 mol/L HCl), produced indirectly via the $^{176}Yb(n,\gamma)^{177}Yb \rightarrow (\beta-) \rightarrow ^{177}Lu$ reaction, was generously donated by Isotope Technologies Garching (ITG, Garching GmbH). In-house-produced ^{177}Lu or purchased from PelkinElmer was also used for the experiments. Aliquots of $^{177}LuCl3$ (15 – 400 MBq, 2 – 48 µL) was mixed with 0.5 mol/L NH_4OAc buffer at pH 7.0, followed by 20 – 50 µL (0.050-0.200 mg) of the conjugates. $^{90}YCl_3$ (555 MBq in 22 µL of 0.05 M HCl) was purchased from PerkinElmer. Aliquots of $^{90}YCl_3$ (28 – 56 MBq, 1-2 µL) were added to 20 -40 µL of 0.5 mol/L NH_4OAc buffer at pH 7.0, followed by 20 – 50 µL (0.050-0.200 mg) of conjugates. Afterward the reaction mixture was incubated at RT or 42 °C for 30 or 60 min for DTPA and DOTA derivatives, respectively.

2.6. Quality control

For stopping the reaction and binding of free radiometal, 1/10 of the total radiolabeling reaction volume of DTPA 0.01 mol/L, pH 6.0, was added and the mixture was incubated for 15 min at room temperature.

Radiometal labelling efficiency and radiochemical purity were determined by thin-layer chromatography performed on SG-ITLC plates (Pall Corporation, USA), using 10% (w/v) ammonium acetate: methanol (1:1) as the mobile phase. SE-HPLC was also employed using TSK-Gel SW 3000 (7.5 × 300 mm, 10 µm, TosoHass) column, with an isocratic mobile phase of NaCl 0.9%/NaN_3 0.05% and a flow rate of 1.0 mL/min. When needed, radiolabeled conjugates were purified from unbound radiometal by size exclusion chromatography on PD-10 column eluted with phosphate buffered saline at 0.05 mol/L.

2.7. Cell Culture

The human cell lines from squamous carcinoma (A431 and SCC25), colorectal carcinoma (SNU-C2B), glioblastoma-astrocytoma (U373 MG), prostate carcinoma (DU145) and the mama carcinomas (HCC 1419 and SK-BR 3) were used in the in vitro experiments. In addition, the human skin fibroblast cell line BJ was used as a control. A431, SK-BR 3 and SCC25 cell line was cultured in DMEM high glucose medium. U373 MG, SNU-C2B, DU145 and BJ cell lines were cultured in RPMI 1640. All media were supplemented with 10% fetal calf serum and Penicilin/Streptomycin (penicillin 100 IU/mL and streptomycin 100 µg/mL). During cell culture and cell experiments (unless otherwise stated), cells were grown at 37°C in incubators with humidified air, equilibrated with 5% CO_2.

2.8. Binding saturation and competitive assays

For saturation binding and competitive experiments A431, SNU-C2B, HCC 1419 and SK-BR 3 cells were cultured ~2 × 10^5cells/well on 12-well-plates (BD Falcon™, Becton-Dickinson, UK) in 1 mL of medium for 24 h prior to the studies. Cells were treated with solutions of different concentrations (100 µL/well, 2.7–120.0 nmol/L) of radiolabeled conjugates for the saturation-binding assay. For the competitive binding assay cells were incubated with 100 µL/well (50 ng, ~75000 cpm/well) of radioimmunoconjugates in cell culture medium and incresing concentrations of unmodified mAbs. After incubating the cells for 3 hours at 4 ºC or 37 ºC, the medium was discarded and cells were washed with ice-cold phosphate buffer solution (PBS). Cells were lysed using 0.5 mL of 0.5 M NaOH containing 5% SDS and radioactivity was measured in an automatic γ or β-counter. The nonspecific binding was studied by adding a 100 times excess of native Nimotuzumab or Trastuzumab to some wells. Triplicate cell dishes were used for each measuring point. For cell counting, a representative parallel sample from the experiments was trypsinized for about 15 min and the cells were counted using a fully automated cell analyzing system (CASY TT, Roche Innovatis AG, Germany). The mean was used as a cell number for all wells. Dissociation constants (Kd), half maximal inhibitory concentration (IC50) and maximal binding sites (Bmax) were estimated from the non-linear fitted curves using GraphPad Prism software (GraphPad Software, Inc., California, USA). The immunoreactivity fraction of the radioimmunoconjugates was determined using the method described by Konishi et. al., based on competitive binding assay using fixed cell concentration.

2.9. Cytotoxicity study

For cytotoxic study A431, SNU-C2B, SCC25, HCC 1419, U373 MG, PC-3 and SK-BR 3 cells were cultured (~2 × 10^5 cells/well) on 12-well-plates for 48 h. Cells were treated with ^{90}Y-DOTA-Bn-hR3/Herc with different DOTA-Bn/mAb molar ratios, Theraloc®, Herceptin™ and $^{90}YCl_3$ for 10 days and cell proliferation was then assessed. At corresponding time point cells were trypsinazed and living cells were counted using CASY TT. The increase in cell number was compared with control samples.

2.10. In vivo studies

As reported previously, animal experiments were performed according to the Animals Ethics Committees of the Faculty of Pharmacy (Charles University, Hradec Kralove, Czech Republic) or Institute of Nuclear Research (Husinec-Rez, Czech Republic).

2.11. Biodistribution of ^{177}Lu-nimotuzumab in mice bearing SNU-C2B xenograft

The *in vivo* studies of ^{177}Lu-DOTA-Bn-h-R3 in tumour model was conducted in female SNU-C2B engraft bearing athymic mice. The mice were injected subcutaneously with SNU-C2B tumour cells (~6 to 8 million cells per tumour in 100 µL of cell culture medium). The tumours were allowed to grow for two weeks before the experiments were performed. Mice were tail vein injected with approximately 100 kBq (50 µL, 10 µg) of ^{177}Lu-DOTA-Bn-h-R3. Preparation with 50-fold excess of p-SCN-Bn-DOTA to modify Nimotuzumab was used. Groups of three animals were anesthetized, bled, sacrificed and dissected at 3, 24, 48, 72 and 144 h after injection. In addition to the tumours, blood, liver, kidney, muscle, stomach and skin were collected, weighed, and measured in a γ-counter. The percentage of injected dose per gram tissue was determined.

2.12. Biodistribution of ^{177}Lu-nimotuzumab in mice bearing A431 xenograft

Mice bearing A431 human epithelial carcinoma xenogratfs were injected intravenously via tail vein with approximately 100 to 280 kBq (100 µL, 10 µg) of ^{177}Lu-DOTA-Bn-h-R3. Groups of three to four animals were anesthetized, euthanized by cervical dislocation, and dissected at 24, 72, 96, 168, 216 and 264 h after injection. In addition, ^{177}Lu-DOTA-Bn-h-R3 conjugate was applied directly into the volume of the tumour tissue for a second group of mice, to study the in vivo behavior of loco regional applied radioimmunoconjugate. Groups of four animals were anesthetized, euthanized by cervical dislocation, and dissected at 24, 72 and 96 h after injection. At the selected time points for each experiment, blood was collected by cardiac puncture and tumours and main organs were collected, weighed, and measured in an automatic γ-counter. The percent of injected dose per gram of tissue were determined using standards representing the injected dose per animal and tumour-to-non tumour (T/nT) tissue ratios were also calculated.

2.13. Statistical analysis

Statistical analysis was performed to compare the results from radiolabeling, in vitro studies and the differences in tissue uptakes among the conjugates using Student's *t*-test ($P < 0.05$) or analysis of variance (One or Two-way ANOVA test) by the software package GraphPad Prism. The ANOVA test was followed by Bonferroni's multiple comparison test ($P < 0.05$). The same approach was used to determine the differences between intravenously and locoregional application.

3. Results and discussion

3.1. Anitbodies modification and radiochemistry

Conjugates with high purity and low-grade aggregation were obtained (Figure 1). The concentration of conjugates ranged from 5.5 – 8.0 mg/mL as determined by Bradford assay. Conjugation reaction gave final chelate to mAbs ratios of 2-4, 9, 12 and 15 for 20:1, 50:1, 100:1 and 150:1 chelate/mAb molar ratios, respectively. For rituximab the conjugation reaction gave 2 – 3 1B4M-DTPA group per mAb molecule.

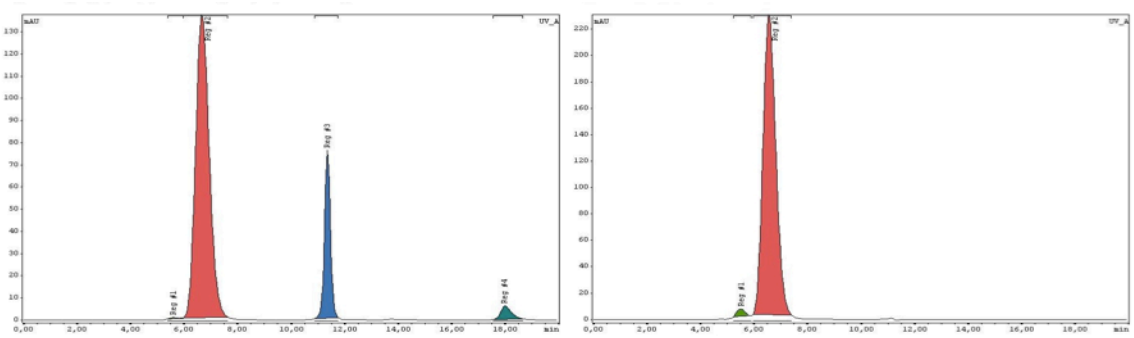
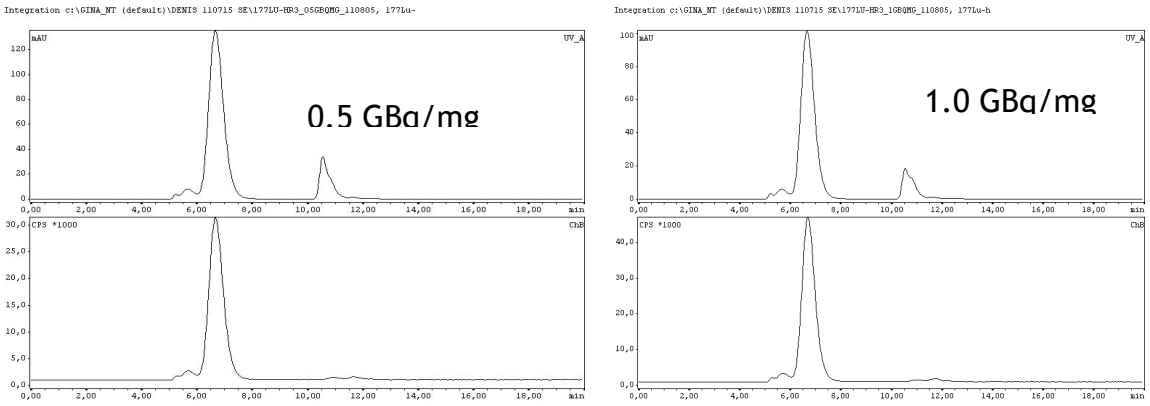
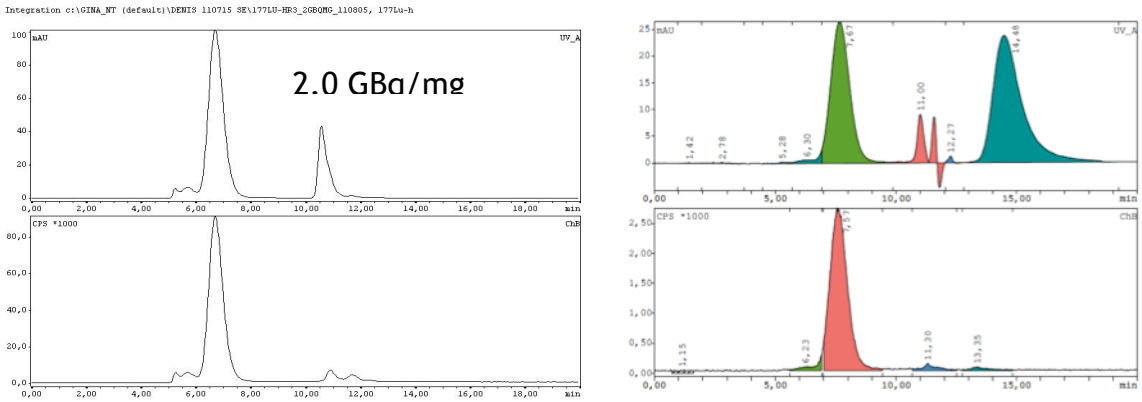


FIG. 1. HPLC profiles of DOTA-mAbs conjugate.

Since ^{90}Yand ^{177}Lu is used for endoradiotherapeutic purpose, this study was undertaken to obtain radioimmunoconjugates with high specific activities. Radiolabeling yield higher than 90% were obtained. Radioimmuconjugates with high radiochemical purity (> 98%) and specific activity up to 2 GBq/mg (Figure 2) were obtained. The quality of the radionuclide and the volume of radiolabeling mixture influenced the radiolabeling efficiency [9]. The radiolabeling yields and specific activities achieved are suitable for radiopharmaceutical preparation. It seems to be that the radioactive concentration, not only in the radionuclide preparation but also in the final reaction mixture, plays an important role in the labelling of mAbs with radiometals.



(a) (b)



(b) (c)

FIG. 2. Radio HPLC profiles of the radiolabeled conjugates where the uppper part corresponds to UV profile where the main peak (left) and the second peak (right) corresponds to the radioimmunoconjugate and DTPA added for the quality control, respectively: a) ^{177}Lu-DOTA-hR3, SA 0.5 GBq/mg. b) ^{177}Lu-DOTA-hR3, SA 1 GBq/mg. c) ^{177}Lu-DOTAhR3, SA 2 GBq/mg. d) ^{90}Y-14BM-Rituximab.

3.2. In vitro studies

Radioimmunoconjugates without significant loss of biological activity were obtained. The binding of the radioimmunoconjugate to the cell lines was receptor specific estimated for the ability to compete with native unmodified mAbs [9-12]. The immunoreactivity fraction of labelled mAbs was higher than 90%. The results showed that the conjugation and radiolabeling did not significantly affect the specifity and affinity of the conjugate.

The results from the cell proliferation assay with ^{90}Y-mAbs conjugates, unmodified mAbs and ^{90}Y showed that ^{90}Y-mAbs conjugates increased cell growth proliferation compared with unmodified mAbs and ^{90}Y (Figure 3). The cell proliferation study shows that ^{90}Y-DOTA-Bn-h-R3 conjugates, like nimotuzumab, selectively binding to cells which moderate to high EGFR expression due to the necessity of bivalent binding in order to have stable attachment to the cell surface [13]. The increased cell proliferation inhibition of ^{90}Y-DOTA-Bn-conjugates over unmodified mAbs or ^{90}Y alone in treated cells might be explained as due to the crossfire effect of the ^{90}Y radiation and its residualization.

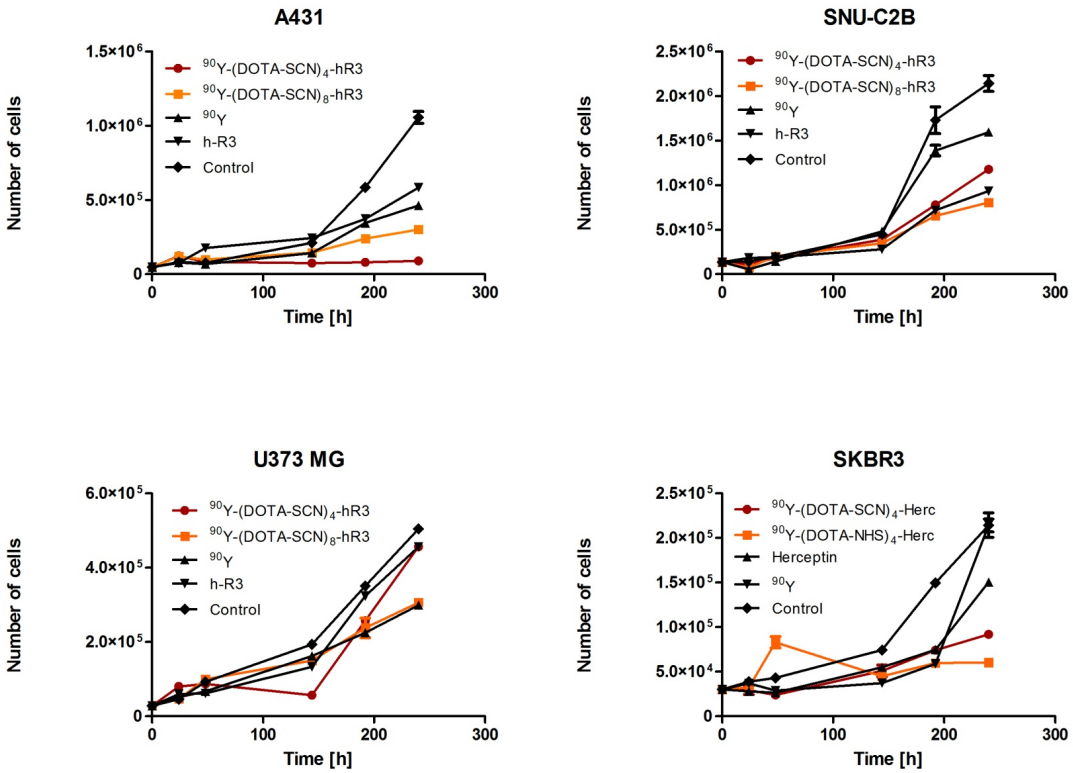


FIG. 3. Cell growth inhibition assay

In a previous study [14], Trastuzumab labeled with ^{111}In showed 1.2 fold higher toxicity in cultured SKBR3 cells than unlabeled trastuzumab whereas in a recent study[15] Trastuzumab labelled with ^{177}Lu showed 4.5-fold higher toxicity in cultured SKBR3 cells than unlabeled trastuzumab. In this study, it was observed that ^{90}Y-(DOTA-NHS)8-9-Herceptin and ^{90}Y-(DOTA-Bn)8-9-Herceptin showed 3.0 and 1.7-fold higher toxicity, respectively, than unlabeled trastuzumab. Consequently, target RIT with ^{90}Y (h-R3 or Trastuzumab) could be a promising approach to increase the apoptotic effect of h-R3 and Trastuzumab in cells with intermediate to high EGFR expression. Due to energy and maximal tissue penetration of the beta particles emitted by ^{90}Y and the results of the citotoxicity studies, ^{90}Y-hR3/Herc might be suitable for treatment of a large diameter solid tumours.

3.2. In vivo studies

The results of the biodistribution study of ^{177}Lu-DOTA-Bn-h-R3 in mice with SNU-C2B tumours xenograft, previously reported [15], showed significant uptake in blood, liver, kidneys, tumours and the skin connected to tumours. The maximum and the average uptake of the radioimmunoconjugate for the SNU-C2B tumours were 10.0 ± 1.9 % ID/g. In this study, tumours uptake of ^{177}Lu-DOTA-Bn-h-R3 was higher than reported by Alejo Morales-Morales et al. [16], Velikyan et al.[17] , S.-Y.Lee et al. [18] and similar as reported by D. E. Milenic et al. [19].

The biodistribution studies of ^{177}Lu-(DOTA-Bn)n-h-R3, n = 4-5 were conducted in A431 human epidermal carcinoma xenografts in Balb/c nude mice. Tumor uptake reached maximum value of 22.4 ± 3.1 %ID/g at 72 h. From 72 to 216 hours the average tumor uptake was 18.3 ± 0.3 %ID/g. Bone uptake was low with maximum value of 2.7 ± 1.1 %ID/g at 24h and an average of 1.6 ± 0.5 from 72 to 264 h. Tumor to non tumours tissues

ratios reached maximum values of 2.6, 2.4, 6.1, 6.6, 23.5 and 17.5 for blood, liver, spleen, kidneys, muscle and bone, respectively. When ^{177}Lu-(DOTA-Bn)n-h-R3, n = 4-5 conjugate was applied locoregionally to A431 xenografts in Balc/c nude mice, tumour uptake was significantly higher at 24 and 72 h than those observed for intravenous application ($P < 0.001$). Tumour uptake peaked 32.7 ± 2.9 %ID/g at 72 h. Locoregional application of the ^{177}Lu-DOTA-Bn-h-R3 conjugate showed T/nT ratios 4.6, 9.8, 10.5, 9.8 and 11.7 times higher for blood, liver, spleen, lungs and kidneys, respectively than those calculated for i.v. application. At 72 h the T/nT ratios mentioned above were two times higher for LR than IV application. In this study, the tumour uptake values and tumour/non tumour ratios are higher than those previously reported for h-R3 and the parental murine mAb labelled with ^{99m}Tc and ^{90}Y.[20, 21].

4. Conclusions

Radioimmunoconjugates with high radiolabeling yield, radiochemical purity and specific activity were obtained without significant loss in its biological activity. Cytotoxicity studies showed the potential of the radioimmunoconjugates over unmodified monoclonal antibodies to inhibit cell proliferation in cell lines with intermediate to high EGFR/HER-2 expression. ^{177}Lu/^{90}Y-DOTA-h-R3/Trastuzumab may have a potential for further evaluations as a radiopharmaceutical for RIT of EGFR overexpressing tumours.

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Preliminary results in animal models with ^{177}Lu- and ^{90}Y labelled monoclonal antibodies and peptides

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Abstract. The major work carried-out in Hungary focused on in vivo testing of radiopharmaceutical applicant molecules. Elements of a complete biological evaluation system were published in earlier IAEA-coordinated projects. In this present period beyond the known normal and pathological animal distribution-, and radiotoxicological studies we established several double xenografted Nude mice models that are available to image and quantify the specific and non-specific binding of tumor-seeking radioligands. Spontaneously occurring canine tumors (eg.: canine insulinomas and B-cell lymphomas) were also selected and their usefulness proved in therapeutical radiopharmaceutical research. Selection of the referred animal patients based on PET/CT or SPECT/CT whole body hybrid images (^{68}Ga-DOTA-TATE, ^{99m}Tc-HYNIC-TATE, and FDG PET/CT) and immunohistochemical evaluation of biopsy specimens were carried-out. We also performed pilot studies for enhancing the radiopharmaceutical uptake in tumor xenografts by the use of locoregional electromagnetic hyperthermia (Oncothermia®). This complete animal testing method is capable to provide important preclinical data including safety-, kinetic-, excretion-, internal dosimetry-, and efficacy results that are assisting the decision making process for investigators. Hungary still offers its animal testing capacities for all other interesting participating countries.

1. Introduction

Numerous regulatory peptide receptors are overexpressed in some specific cancers. The specific receptor binding property of peptides can be exploited by their labelling with a radionuclide and their use as carriers to guide the radioactivity to the tissues expressing their specific receptors. During the past decade, radiolabelled receptor-binding peptides have emerged as an important class of radiopharmaceuticals for tumour diagnosis and therapy. The first and most successful imaging agents to date are somatostatin analogues, which are routinely used for somatostatin receptor scintigraphy. Peptide receptor radionuclide therapy (PRRT) with 90Y-DOTA-octreotide and 177Lu-DOTA-octreotate in neuroendocrine tumours (NETs) results in symptomatic improvement, prolonged survival, and enhanced quality of life. The results in terms of tumour regression are very encouraging with few and mostly mild side effects when patients are carefully selected and kidney protective agents are given. Nevertheless much profit can be gained from improving the available strategies and developing new strategies. Peptides such as minigastrin, glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK), bombesin (BN)/gastrin-releasing peptide (GRP), substance P, neurotensin (NT), neuropeptide Y (NPY) and RGD peptides are promising for PRRT and currently under preclinical and clinical development [7].

Radioimmunotherapy (RIT) combines the targeting advantage of a monoclonal antibody with the radiosensitivity of non-Hodgkin lymphoma (NHL) cells. The unlabeled antibodies have indeed added efficacy, but many patients still relapse. One method of enhancing the cytotoxic potential of monoclonal antibodies is to attach them to a radionuclide to form a radioimmunoconjugate (RIC). The use of an RIC has the advantage of

targeting not only the cell to which the antibody is bound but also the surrounding tumor cells and microenvironment.

The similarities between dog and human cancers are increasingly being recognized and utilized by biomedical researchers and pharma-, equipment industry. The publicly available canine genome has initiated comparative genomics studies too. Pet owners are highly motivated to seek novel options for management of cancer in their pets, especially if conventional treatments do not meet their expectations. A pet owner's decision to pursue an investigational treatment is often influenced by the risks associated with this therapy compared to conventional therapy, as well as their expectations for outcomes and reduced costs for care provided by an investigational trial. Additionally, many pet owners are motivated by the opportunity to contribute to the advancement of cancer treatment for future human and canine patients. Experience of investigators using these tumors has shown that animal owners and referring veterinarians are most cooperative in reasonable, humane approaches to experimental cancer therapy. From the researcher's point of view the advantages of spontaneous animal tumors in larger animals include the relatively large tumor volumes and correspondingly larger sizes of treatment fields. Longer follow-up times are also generally possible. Clinical data obtained through serial monitoring can be obtained in addition to the data of local tumor control and normal tissue response. Animals with spontaneous tumors often have concomitant diseases similar to those encountered in humans, which may or may not complicate the management of the tumor. In these dogs, cancers develop naturally in the context of an intact immune system and with a syngeneic host and tumor microenvironment. Similar environmental, nutrition, age, sex, and reproductive factors lead to tumor development and progression in human and canine cancers. They share similar features such as histological appearance, tumour genetics, biological behavior, molecular targets, therapeutic response, and unfortunately, acquired resistance, recurrence, and metastasis. Thus all the above listed facts make the referred, spontaneously occurring canine tumors maybe the best model of human malignancies.
Similarly to several other neuro-endocrine malignancies canine insulinoma shows very relevant similarities human insulinoma including the overexpression of somatostatin (especially SSR-2a subgroup) receptors. B-cell originated malignant canine lymphomas proved to be the best model of human non-Hodgkin lymphomas in several studies. As human B-cell lymphomas also in dogs malignant cells overexpress CD-20 antigen in the cell membrane presebting a convenient target for immuno- imaging and therapy in both species.

2. Preliminary results

2.1. Results on double-xenografted nude mice

More recently we realized that several advantages are provided if the same immunosupressed animal being inoculated with two different tumor cell lines. The main advantage is that it is available to compare, image and quantify the differencies between specific and non-specific tumor uptakes within the same living organism. Other characteristics e.g., blood-flow, glucose metabolism, hypoxic-, apoptotic regions of tumors could be also evaluated in the different xenografts which could provide also usefull data to understand unawaited results.

TABLE 1. Summary of characteristics of several double-xenografted nude mouse models

No	Left thigh	Right thigh	Tested radiocompounds
1	INS-1 (SSR2 +++ but folate -)	HeDe (SSR2a - but folate +++)	^{68}Ga-DOTA-TATE ^{99m}Tc-HYNIC-TATE ^{177}Lu-DOTA-TATE ^{68}Ga-folate targeting colloid ^{99m}Tc-folate targeting colloid

2	INS-1 (SSR2 +++ but folate -)	NRIRR/ca/09 (SSR2a +++ but folate-)	^{68}Ga-DOTA-TATE ^{99m}Tc-HYNIC-TATE ^{177}Lu-DOTA-TATE
3	Raji (CD20+++ but)	HeDe (SSR2a - but folate +++)	^{177}Lu-Rituximab ^{68}Ga-folate targeting colloid ^{99m}Tc-folate targeting colloid
4	A431 EGFR+++ but SSR2-)	INS-1 (SSR2 +++ but folate -)	^{177}Lu-hR3 ^{177}Lu-hR3 ^{68}Ga-DOTA-TATE ^{99m}Tc-HYNIC-TATE ^{177}Lu-DOTA-TATE
5	WRO (TSHR+++ but SSR2-)	INS-1 (SSR2 +++ but folate -)	^{99m}Tc-super TSH analogue ^{68}Ga-DOTA-TATE ^{99m}Tc-HYNIC-TATE ^{177}Lu-DOTA-TATE

In preliminary tests we found that radiopharmaceutical uptake in xenografts that overexpress the specific target could be 1.5 -18 times higher than in contralateral negative tumors.

If the Nude mice is inoculated with two same cell lines the model is available to measure the effects of different locoregional treatments. In our pilot studies locoregional electromagnetic hyperthermia (Oncothermia®) increased the blood-flow with 10-15% and the specific uptake upto 30% in the treated tumor compared to the other contralateral, non-treated xenograft. More studies are needed to prove the significance of this above observation.

2.2.1. Results on the referred spontaneously occurring insulinomas

Spontaneously occurring dog patients with a suspection of insulinoma were referred to our Institute for final diagnosis and treatment if possible. Suspection was made on hypoglycaemia and hyperinsulinoma measured in blood and serum samples. The diagnosis was made by blood hematology, biochemical panel, repeated glucose and insulin measurements and whole body hybrid imaging was also carried-out for localizing the tumors, stadium based diagnosis and proving the in vivo presence of target (SSR2). Dogs were injected either by ^{99m}Tc-HYNIC- TATE (SPECT/CT) or ^{68}Ga-DOTA-TATE (PET/CT) and inclusion criteria were the clear focal localisation of radioligands in at least 1 pancreas laesion and more than one hepatic lesions. Altogether 14 dogs fullfilled the criterias but only 9 owners choosed the offered systemic radioimmunotherapy.

The included dogs were hospitalized and treated by a single injection of ^{177}Lu-DOTA-TATE peptide at a dose of 100 MBq/ bwkgs. Hematological and biochemical parameters and clinical signs (especially lymph node sizes) were monitored weekly at the first month, then at 3, 6, 9 and 12 month after therapy.

TABLE 2. Efficacy results of 9 referred dogs with insulinoma treated by a single injection of ^{177}LuDOTA-TATE in a dose of 100 MBq/ bwkgs.

No	Dog	Diagnostic	3ms FU	6ms FU	9ms FU	12ms FU
1	7ys, intact male,36 kg	^{99m}Tc HYNIC-TATE,SPECT/CT	Symptom free	Symptom free	Symptom free	Symptom free

2	9ys, castrated male, 39 kg	^{99m}Tc HYNIC-TATE,SPECT/CT	Symptom free	Symptom free	Symptom free	Symptom free
3	11 ys , spayed female, 42 kg	^{99m}Tc HYNIC-TATE,SPECT/CT	Symptom free	Symptom free	Symptom free	Symptom free
4	12 ys spayed female, 8 kg	^{99m}Tc HYNIC-TATE,SPECT/CT	Symptom free	Symptom free	Symptom free	Symptom free
5	8 ys , intact female, 22 kg	^{99m}Tc HYNIC-TATE,SPECT/CT	Symptom free	Symptom free	Recurrent disease	Euthanasia
6	9ys, intact male,13 kg	^{99m}Tc HYNIC-TATE,SPECT/CT	Symptom free	Symptom free	Symptom free	Symptom free
7	10 ys , spayed female, 31 kg	^{68}GaDOTA-TATE PET/CT	Symptom free	Symptom free	Symptom free	Recurrent disease
8	12 ys , spayed female, 51 kg	^{68}GaDOTA-TATE PET/CT	Symptom free but cardiac failure	Symptom free but cardiac failure	Died because of cardiac/ renal failure	NA
9	8 ys, spayed female , 17 kg	^{68}GaDOTA-TATE PET/CT	Symptom free	Symptom free	Symptom free	Symptom free

Eigth out of 9 dogs tolerated well the treatment no side effects were observed. The only dog we lost 7 months after completing the therapy was a large sized, elderly, obes dog having a subclinical cardiac insufficiency that was only detected 8 days after initiating the treatment. This particular dog started to loose weight, developed renal- and liver insufficiency and was euthanized on the owner's request because of progressing cardiac failure and renal/liver problems. Autopsy revealed severe cardiac enlargement, cardiomyopathy, renal atrophy, hepatomegalia, intra- and perilobal fattic hepatodystrophia.

In all the 9 treated dogs we realized a fast normalization of glucose and insuline levels, 5-10 days after radiopharmaceutical application the glucose and insuline concentrations were found within the normal ranges. The symptoms (hypoglycaemia and hyperinsulinoma) recurred in one dog 9 months and in another dog 12 months after completing the treatment but 6 out of 9 dogs were symptom free even 12 month after the treatment.

This preliminary data are very promising especially because the disease in this stage is inoperable in dogs as well and because that conventional chemotharapy known to provide only questionable results in dogs too.

2.2.2. Results on the referred spontaneously occurring malignant canine lymphomas

Spontaneously occurring dog patients with a suspection of malignant lymphoma were referred to our Institute for final diagnosis and treatment if possible. Suspection was made on chronicly enlarged lymph nodes with no known intermittant infections or parasitic diseases. The diagnosis was made by blood hematology, biochemical panel, and cytology (immunohistochemistry on CD-20 antigen) and whole body FDG PET/CT imaging was also carried-out for localizing the tumors, and give the stadium-based diagnosis. The inclusion criteria were : the involvement of more than 10 lymph nodes, liver and spleen but not the bone marrrow in the entire body (Stage 4a) and the immunohistochemical prove of CD20 antigen presence from a removed lymph node. Altogether 6 dogs fullfilled the criterias and 4 owners choosed the offered systemic radioimmunotherapy.

The included dogs were hospitalized and treated by a single injection of ^{90}Y-anti CD20 (Zevalin) antibody at a dose of 15 MBq/ bwkgs. Hematological and biochemical parameters and clinical signs (especially lymph node sizes) were monitored weekly at the first month, then at 3, 6, 9 and 12 month after therapy.

TABLE 3. Efficacy results of 4 referred dogs with stage 4a B-cell lymphoma treated by a single injection of 90Y-anti CD20 (Zevalin®) in a dose of 15 MBq/ bwkgs.

No	Dog	Diagnostic	3ms FU	6ms FU	9ms FU	12ms FU
1	7ys, intact female,30 kg	FDG, PET/CT	Symptom free	Symptom free	Symptom free	Recurrent disease
2	9ys, castrated male, 31 kg	FDG, PET/CT	Symptom free	Symptom free	Symptom free	Symptom free
3	11 ys , spayed female, 27 kg	FDG, PET/CT	Symptom free	Symptom free	Symptom free	Symptom free
4	12 ys intact male, 8 kg	FDG, PET/CT	Symptom free	Symptom free	Recurrent disease	Recurrent disease

All the 4 dogs tolerated well the treatment no severe side effects occured. Between 3-10 days postapplication an intermittant increase of renal parameters (carbamid, creatinine, phosphore) in each dogs were observed but they were normalized without treatment.

In all the 4 treated dogs we realized a fast decrease in lymph node sizes, 5-10 days after radiopharmaceutical application the lymp node sizes normalized. The symptoms recurred in one dog 9 months and in another dog 12 months after completing the treatment but 2 out of 4 dogs were symptom free even 12 month after the treatment.

Collected data of ^{90}Y-anti CD20 (Zevalin) monoclonal antibody will be available for later ^{177}Lu-conjugated-Rituximab comparison.

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INDIA

Development of therapeutic radiopharmaceuticals based on ^{177}Lu and ^{90}Y labelled monoclonal antibodies and peptides

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1. Introduction

The objective of the present Coordinated Research Program (CRP) is to develop target-specific therapeutic radiopharmaceuticals based on ^{177}Lu- and ^{90}Y-labeled monoclonal antibodies and peptides. Radioimmunotherapy (RIT) or treatment of cancerous lesions over-expressing tumor-specific antigens by radiolabeled antibodies is an effective modality to combat a wide variety of cancers. Two radiolabeled monoclonal antibodies, namely ^{90}Y-labeled anti-CD20 antibody (Zevalin®) and ^{131}I-labeled anti-CD20 antibody (Bexxar®) are regularly used to treat non-Hodgkin's lymphoma [1,2]. Several other monoclonal antibodies labeled with a variety of β^- and $\propto$-emitting radionuclides are presently undergoing different stages of clinical trials [3,4]. Similarly, peptides labeled with suitable therapeutic radionuclides could be effectively used to treat cancers over-expressing specific type of receptors. Towards this, the utilization of ^{177}Lu-labeled DOTA-TATE to treat the patients suffering from neuro-endocrine cancers over-expressing somatostatin receptors needs special mention [5,6]. Apart from these, other peptides, such as VIP, bombesin, gastrin releasing peptides, minigastrin, substance P, RGD have been labeled with different therapeutic radionuclides for developing agents for targeted tumor therapy [7,8].

Although several β^- and a few $\propto$-emitting radionuclides have been used for the development of targeted therapy agents based on antibodies and peptides, ^{177}Lu and ^{90}Y could be the two best possible isotopes for developing such agents [9]. Multi-Curie levels of ^{177}Lu, with adequately high specific activity, could be produced by simple direct neutron capture route using enriched (in ^{176}Lu) Lu_2O_3 target in medium flux research reactors and supplied without much decay loss owing to its comparatively longer half-life [9,10]. On the other hand, availability of ^{90}Sr/^{90}Y radionuclide generator system capable of producing clinical grade ^{90}Y in a hospital radiopharmacy will ensure the uninterrupted supply of ^{90}Y-based radiopharmaceuticals over a long period [9,11]. The work carried out in the Radiopharmaceuticals Division (RPhD), Bhabha Atomic Research Centre (BARC) following RCM-1 of this CRP is outlined in the present report. Herein, we discuss our efforts toward production of multi-Curie levels of ^{177}Lu and development of suitable ^{90}Sr/^{90}Y radionuclide generator, preparation of antibody-bifunctional chelating agent (BFCA) conjugate and subsequent optimization of the labeling protocols for the preparation of ^{177}Lu and ^{90}Y-labeld antibodies, such as, MabThera® and Reditux®. The report also describes our effort towards the development of a DOTA-TATE cold kit, suitable for the preparation of therapeutic doses of ^{177}Lu-DOTA-TATE for human administration.

2. Production of ^{177}Lu

Radiopharmaceuticals Division, Bhabha Atomic Research Centre has initiated research on the development of ^{177}Lu-based therapeutic agents as early as 2000 and started supplying this radionuclide for clinical applications from 2007. With the introduction of ^{177}Lu-DOTA-TATE therapy in India in 2007 for the treatment of patients suffering from various types of neuro-endocrine cancers; the demand for adequately high specific activity ^{177}Lu [$T_{1/2}$ = 6.73 d, $E_{\beta(max)}$ = 497 keV, E_γ = 208 keV (11%), 113 keV (6.4%], suitable for the preparation of receptor-based therapeutic agents, has shown constant and steady increase [12]. In order to cater this increased

requirement, production facilities at the Radiopharmaceuticals Division have recently been augmented. At present, high specific activity clinical grade (sterile and pyrogen free) ^{177}Lu is being regularly produced at our Institute and supplied to several nuclear medicine centres across our country for the preparation of therapeutic radiopharmaceuticals. Starting from a modest production of ~6.5 Ci (~241 GBq) and supplying ~2.7 Ci (~100 GBq) (11 consignments) of ^{177}Lu in 2007, we have produced and supplied more than 100 Ci (3.7 TBq) and 50 Ci (1.85 GBq) (75 consignments) of ^{177}Lu, respectively to various nuclear medicine centres of our country till the end of August, 2012 (Figure 1). The frequency of production has also been augmented from once in a month to thrice in a month to cater the need of the nuclear medicine centres.

At present, high specific activity clinical grade ^{177}Lu is produced by irradiating enriched (82% in ^{176}Lu) Lu_2O_3 target at a thermal neutron flux of 9×10^{13}-1×10^{14} n/cm^{-2}.s^{-1} in DHRUVA reactor of our Institute for a period of 3-4 weeks. Following irradiation, the target is dissolved in supra-pure 0.01 M HCl by gentle warming inside a lead-shielded plant. The resultant solution thus obtained is evaporated to near-dryness and subsequently reconstituted in ultra-pure water. The evaporation and reconstitution steps are repeated a few times. Subsequently, the preparation is allowed to pass through the Millipore® filter and dispensed as per the need of the customers.

Assay of total activity produced was primarily done by using a pre-calibrated ion-chamber and subsequently reconfirmed by gamma-ray spectrometry carried out using a HPGe detector coupled with a 4 K multichannel analyzer (MCA). The specific activity of ^{177}Lu produced at the end-of-bombardment (EOB) is observed to be between 814-1295 GBq.mg^{-1} (22-35 Ci.mg^{-}1). The radionuclidic purity of ^{177}Lu is determined by analyzing the gamma-ray spectrum of the appropriately diluted samples of radiochemically processed $^{177}LuCl_3$. The average level of radionuclidic impurity burden in ^{177}Lu due to ^{177m}Lu was found to be 3.7-5.5 kBq of ^{177m}Lu / 37 MBq of ^{177}Lu (100-150 nCi / 1 mCi) at EOB, which indicates that ^{177}Lu has a radionuclidic purity of ~99.98% at the time of supply [10,12]. The radiochemical purity of $^{177}LuCl_3$ is determined by employing paper chromatography technique using normal saline as the eluting solvent and found to be more than 98% at the time of supply.

The chemical purity of ^{177}Lu is another important criterion, particularly when the radionuclide is to be used for the preparation of target-specific agents. It has been documented that presence of chemical impurities such as, Ca, Zn, Fe, Pb, Al, Cu adversely affects the radiolabeling yield of such agents and therefore the level of these impurities should be as low as possible in the processed ^{177}Lu [12]. The levels of chemical impurities present in the ^{177}Lu supplied from our Institute is determined by employing inductively coupled plasma - atomic emission spectroscopy (ICP-AES) and found to be below acceptable limits of such impurities [12].

We have also developed suitable method for the production of no-carrier-added (NCA) ^{177}Lu [theoretical specific activity 4.07 TBq/mg (110 Ci/mg)] which can be produced following the indirect production route involving the neutron irradiation of enriched (99% in ^{176}Yb) Yb_2O_3 target (σ = 2.4 b). This leads to the production of ^{177}Yb ($T_{½}$ = 1.9 h), which subsequently undergoes β^- particle emission to produce ^{177}Lu. A novel two-step electrochemical pathway for the separation of NCA ^{177}Lu from irradiated Yb target has been developed at our end [13]. The method is based on two subsequent electrolysis processes wherein the first separates ^{177}Lu from the bulk of irradiated Yb target and the second one purifies ^{177}Lu by removing the trace level of Yb impurities present in the separated ^{177}Lu. This process provides NCA ^{177}Lu with >99.99% radionuclidic purity and an overall separation yield of ~99% within 3-4 h. The Hg content in the product was determined to be <1 ppm thus making it suitable for various kinds of biomedical applications [13]. A suitable method was also developed to recover the Yb from the ytterbium amalgam to make this production route economically more viable. The method is scaled up to produce 37 GBq (1 Ci) of ^{177}Lu activity and has the potential to be utilized for regular production of NCA ^{177}Lu.

Lu-177 obtained via the indirect route being NCA in nature has very high specific activity and is free from ^{177m}Lu ($T_{½}$ = 160.5 d), whose presence as radionuclidic impurity, is inevitable in ^{177}Lu produced via the direct neutron capture route. However, the production of ^{177}Lu through indirect route is comparatively tedious, less economic and difficult to scale up without high degree of automation [12]. Therefore, this method has not been adopted for regular production of ^{177}Lu in India. On the contrary, as ^{177}Lu with sufficiently high specific activity and in adequate quantity could be easily produced by employing direct neutron capture route, regular production and supply of ^{177}Lu from BARC to nuclear medicine centres is based on this route of production.

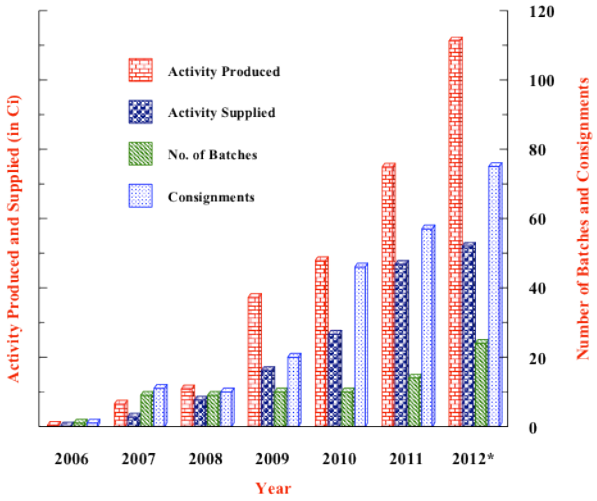


FIG. 1. Growth of ^{177}Lu production and supply in India (updated August 2012).

3. Production of ^{90}Y

Yttrium-90 [$T_{1/2}$ = 64.1 h, $E_{\beta(max)}$ = 2.27 MeV, no γ] is a pure β^- emitter, which has well-established applications in targeted radiotherapy. Yttrium-90 could be obtained either by direct neutron irradiation of natural yttrium target i.e. $^{89}Y(n,\gamma)^{90}Y$ or from a $^{90}Sr/^{90}Y$ radionuclide generator system. However, as the cross-section of $^{89}Y(n,\gamma)^{90}Y$ is very low (σ = 1.28 mb), only low specific activity ^{90}Y could be obtained by this method and hence, it has a limited interest [9]. Therefore, for obtaining large quantities of ^{90}Y for therapeutic applications $^{90}Sr/^{90}Y$ radionuclide generator is preferred. It is reported that, a few thousand Curies of ^{90}Y could be obtained from only 1 Ci (37 GBq) of ^{90}Sr over several years and such a generator can be eluted twice in a week to obtain 450-500 mCi (16.65-18.5 GBq) with each elution [14]. It is also documented that supplementing the parent radionuclide activity by adding about 10% of ^{90}Sr i.e. with 100 mCi (3.7 GBq) in every 4-5 years will be adequate to keep the ^{90}Y supply constant over a very long period [14]. Therefore, effort was directed to develop a $^{90}Sr/^{90}Y$ radionuclide generator system suitable of producing clinical grade ^{90}Y for radiotherapeutic applications.

Working in this direction, we have developed a novel electrochemical method for the separation of ^{90}Y from the bulk of ^{90}Sr [14]. This methodology has been transferred to Isotope Technologies, Dresden, Germany through International Atomic Energy Agency (IAEA) for the development of commercial $^{90}Sr/^{90}Y$ generator. The effort has culminated in the construction of 'Kamadhenu', a fully computer controlled automated $^{90}Sr/^{90}Y$ generator system, capable of producing ^{90}Y suitable for radiotherapeutic applications [14,15]. The generator is based on two-step electrochemical process and capable of separating clinical grade ^{90}Y from the bulk of $^{90}Sr(NO_3)_2$ feed solution. The first electrolysis is performed for 90 min at pH 2-3 at a potential of -2.5 V with 100-200 mA current using platinum electrodes while the second electrolysis is performed for 45 minutes in 0.01 N HNO_3 medium at a potential of -2.5 V with 100 mA current. In the second step, the cathode from the first electrolysis containing ^{90}Y is used as the anode along with a fresh circular platinum electrode as the cathode. The

^{90}Y deposited on the circular cathode after the completion of second electrolysis step is dissolved in 0.1 N HCl to obtain ^{90}Y chloride which is suitable for the preparation of ^{90}Y-labeled agents for biomedical applications. The recovered ^{90}Y had a high radionuclidic purity with only 30.2±15.2 kBq (817±411 nCi) of ^{90}Sr per 37 GBq (1 Ci) of ^{90}Y (0.817±0.411 ppm) is detected [14]. Some of the major advantages of this generator system are the low operational cost, generation of very low volume of radioactive wastes, lesser possibilities of metallic contamination in the eluted ^{90}Y and the possibility of using the ^{90}Sr repeatedly. This generator is presently being used in a few countries. Though 'Kamadhenu' is not acquired in RPhD, the above-mentioned methodology is used for the regular production of ^{90}Y, which is used for the preparation of ^{90}Y-labeled radiopharmaceuticals.

4. Preparation of ^{177}Lu-labeled Rituximab

Non Hodgkin's lymphoma is a type of malignancy of the lymphoid system and is mainly derived from B-lymphocytes. Most B-cell lymphomas express CD20, making it a suitable target antigen for the preparation of radiolabeled monoclonal antibodies. By linking anti-CD20 monoclonal antibodies to an appropriate therapeutic radioisotope, sites of disseminated disease can be targeted effectively. Rituximab is a chimeric antiCD20 B-cell specific monoclonal antibody which is an approved drug for the treatment of low-grade non Hodgkin's lymphoma. Therefore, rituximab labeled with a therapeutic radionuclide could be envisaged as a suitable agent for radioimmunitherapy.

At the time of initiation of this work, we have the accessibility to very limited quantity of Mabthera®, which prompted us to standardize the protocol of preparation of radiolabeled antibodies by using immunoglobulin-G (IgG). The experience gained from the preparation of ^{177}Lu-labeled IgG is directly utilized for the preparation of ^{177}Lu-labeled Mabthera® (commercially available from Roche, Switzerland) and Reditux® (commercially available from Dr. Reddy's Laboratory, India) which are described below.

4.1. Preparation and characterization of ^{177}Lu-labeled IgG

Lu-177 labeled IgG was prepared by a two step process involving coupling of IgG with a suitable bifunctional chelating agent (BFCA) followed by radiolabeling of the BFCA-IgG conjugate. The process followed is described below in brief.

4.1.1. Preparation and purification of p-NCS-benzyl-DOTA-IgG conjugate

In order to facilitate labeling of IgG with ^{177}Lu, it is necessary to couple it with a suitable BFCA. As decided in the previous meeting, *p*-NCS-benzyl-DOTA (*p*-NCS-benzyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) was chosen as the BFCA of choice for the present study owing to its ability to form complexes with lanthanides with high thermodynamic stability and superior kinetic inertness [16]. The condition for BFCA-IgG labeling was standardized by preparing the conjugate using different molar ratios of IgG and BFCA and labeling the conjugates, thus obtained, with ^{177}Lu. The radiolabeling yield of the ^{177}Lu-labeled conjugates were determined by the protein precipitation method which involves precipitation of the protein in the reaction mixture by trichloroacetic acid followed by the separation of precipitate and supernatant by centrifugation and subsequently counting the activity associated with these layers. It was observed that maximum complexation yield was achieved when minimum ratio of IgG to BFCA was maintained at 1:10. Therefore, for subsequent studies IgG-BFCA conjugate was prepared by incubating 5 mg of IgG (concentration 20 mg.mL^{-1}) with 230 µg of *p*-NCS-benzyl-DOTA (corresponding to molar ratio of 1:10) in Na_2CO_3 buffer (pH=9.5) at 37°C for a period of ~17 h. The conjugate thus obtained was purified by removing free *p*-NCS-benzyl-DOTA present in the reaction mixture by using a pre-packed PD10 column. Purification procedure involved loading of ~2.5 mL of the crude BFCA-IgG conjugate on the column followed by fractional elution using NH_4Oac buffer (pH=5.5).

4.1.2. Preparation and characterization of ^{177}Lu-labeled p-NCS-benzyl-DOTA-IgG conjugate

Radiolabeling of purified *p*-NCS-benzyl-DOTA-IgG conjugate with ^{177}Lu was carried out by incubating 200 µg of the conjugate with $^{177}LuCl_3$ (525 MBq, 14.2 mCi, 0.71 µg Lu) at 37°C for a period of 1 h. Characterization of ^{177}Lu-labeled *p*-NCS-benzyl-DOTA-IgG was carried out using HPLC with silica gel column. The elution profile was monitored by detecting the radioactivity signal using a NaI(Tl) detector coupled with the HPLC system. An isocratic system consisting of 0.05 M phosphate buffer with 0.05% NaN_3 as the eluting solvent was used. It was observed that ^{177}Lu-labeled *p*-NCS-benzyl-DOTA-IgG conjugate eluted with a retention time of 14 min whereas ^{177}Lu-labeled *p*-NCS-Benzyl-DOTA eluted with a retention time of 21 min. A typical HPLC profile showing the elution of ^{177}Lu-labeled *p*-NCS-benzyl-DOTA-IgG conjugate is shown in Figure 2. Under the above mentioned optimized reaction conditions, ^{177}Lu-labeled *p*-NCS-benzyl-DOTA-IgG was obtained with >95% radiochemical purity.

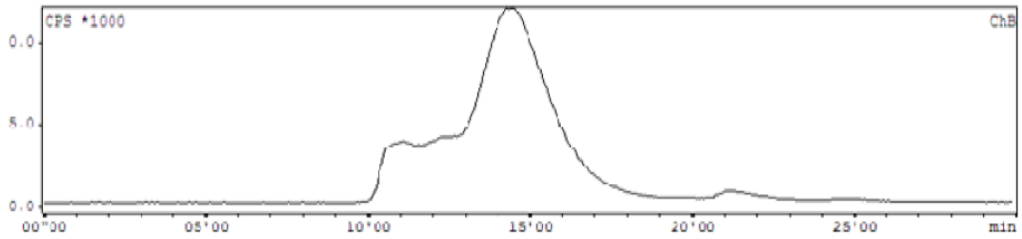


FIG.2. Typical HPLC pattern of ^{177}Lu-labeled IgG.

4.2. Preparation and characterization of ^{177}Lu-labeled MabThera®

Conjugation of MabThera® and *p*-NCS-benzyl-DOTA and subsequent ^{177}Lu-labeling of the coupled product was done by following the protocol developed for the preparation of ^{177}Lu-labeled IgG mentioned above. Lu-177-labeled MabThera® was prepared following a two-step process described below.

4.2.1. Preparation and purification of p-NCS-benzyl-DOTA-MabThera® conjugate

p-NCS-benzyl-DOTA-MabThera® conjugate was prepared by incubating MabThera® with *p*-NCS-Benzyl-DOTA corresponding to molar ratio of 1:10 in Na_2CO_3 buffer (pH=9.5) at 37°C for a period of about 17 h. The conjugate was purified by removing excess BFCA from the reaction mixture using pre-packed PD10 columns. This was achieved by loading the conjugate in the column followed by the elution with NH_4Oac buffer (pH=5.5). Eluants were collected into 1 mL fractions and the fractions containing maximum concentration of *p*-NCS-benzyl-DOTA-MabThera® conjugate [Identified by protein estimation using Lowry (Folin-Ciocalteau) method] were pooled together. The purified conjugate thus obtained was utilized for the preparation of ^{177}Lu-labeled MabThera®.

4.2.2. Preparation and characterization of ^{177}Lu-labeled MabThera®

Lu-177 labeling of *p*-NCS-benzyl-DOTA-MabThera® conjugate was achieved by incubating the conjugate with $^{177}LuCl_3$ (525 MBq, 14.2 mCi, 0.71 µg Lu) at 37°C for 90 min using NH_4Oac buffer (pH~5.5). Characterization of ^{177}Lu-labeled *p*-NCS-benzyl-DOTA-MabThera® conjugate was done by employing HPLC equipped with a gel column and tracking the eluant with NaI(Tl) detector. An isocratic system consisting 0.05 M phosphate buffer with 0.05% NaN_3 was used as the mobile phase. The peak corresponding to ^{177}Lu-labeled antibody-BFCA conjugate was observed at 14 min while uncomplexed $^{177}LuCl_3$ eluted within the void volume. A typical HPLC

profile of ^{177}Lu-labeled *p*-NCS-Benzyl-DOTA-MabThera® is shown in Figure 3. Lu-177-labeled *p*-NCS-benzyl-DOTA-MabThera® conjugate was prepared with >95% radiochemical purity under the optimized reaction conditions.

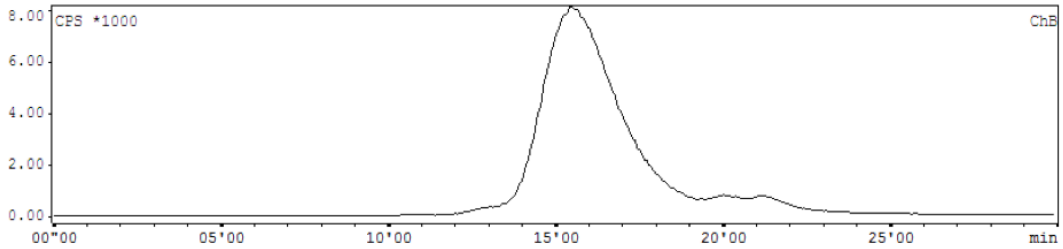


FIG. 3. Typical HPLC pattern of ^{177}Lu-labeled MabThera®.

4.2.3. Determination of average number of BFCAs per MabThera® molecule

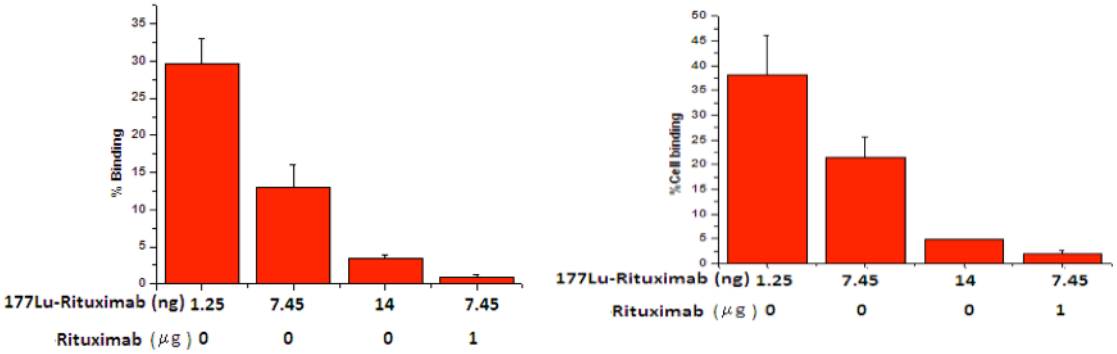
Attempt was made to determine the average number of BFCAs attached per MabThera® molecule in *p*-NCS-Benzyl-DOTA-MabThera® conjugate following the reported method [17]. For this, the conjugate (100 µg) was incubated with ^{177}Lu (74 MBq, 2 mCi) containing excess ^{176}Lu (1.17 µg, 10 times molar excess of antibody-BFCA conjugate) at 37°C for 1 h and then subsequently allowed to react with DTPA (diethylenetriaminepentaacetic acid, taken in equimolar ratio with ^{176}Lu i.e. 2.6 µg) for a period of 30 min at room temperature. Reaction mixture thus obtained was injected into HPLC column and the areas under peaks corresponding to ^{177}Lu-labeled *p*-NCS-benzyl-DOTA-MabThera® conjugate as well as ^{177}Lu-labeled DTPA were determined. The average number of BFCAs attached per MabThera® molecule was determined from these data and was found to be 6.

4.2.4. Stability of ^{177}Lu-labeled MabThera®

The in-vitro stability of ^{177}Lu-labeled MabThera® was checked by incubating the radiolabeled antibody at room temperature and determining its radiochemical purity at different time intervals by employing the quality control techniques mentioned above. It was observed that ^{177}Lu-labeled MabThera® retained its radiochemical purity >95% till 3 d, upto which the study was continued.

4.2.5. Biological evaluation of ^{177}Lu-labeled MabThera®

Raji cells (Burkitt's lymphoma), which express CD20 antigen on their surface, were used for *in-vitro* binding studies of ^{177}Lu-labeled MabThera®. Cells were grown to confluence in RPMI-1640 medium containing 10% fetal calf serum. After harvesting, 1×10^6 cells were incubated with ^{177}Lu-labeled MabThera® for 2 h at 37°C and 4°C. After incubation, the cells were washed thrice with 5 mL of Dulbecco phosphate buffer (DPBS) and centrifuged at 2000 rpm for 6 min at room temperature. The supernatant was decanted and the pellet was measured for radioactivity. Cell binding studies were performed with 1.25, 7.45 and 14 ng of ^{177}Lu-labeled MabThera® and a maximum of ~30% and ~37% binding were recorded when 1.25 ng of radiolabeled antibody were incubated at 37°C and 4°C temperature, respectively. Inhibition studies were carried out to confirm the specificity of binding by incubation of same number of cells with 7.45 ng of ^{177}Lu-labeled MabThera® along with 1 µg of cold MabThera® under similar experimental conditions. Significant inhibition of bindings was observed at both the incubation temperatures which show the specificity of the radiolabeled antibody towards the CD20 antigen. The results of the cell binding studies are shown in Figure 4 (a) and (b).



(a) (b)

FIG. 4. Results of cell binding studies carried out with ^{177}Lu-labeled MabThera® in Raji cell line at (a) 37°C and (b) 4°C temperature.

4.3. Preparation and characterization of ^{177}Lu-labeled Reditux®

Recently we have started a collaborative work with Tata Memorial Centre (TMH), a premier super speciality cancer hospital in India, towards the development of patient doses of ^{177}Lu-labeled rituximab for the treatment of patients suffering from non-Hodgkins's lymphoma. Towards this we have received the bulk supply of Reditux®, which is commercially available rituximab available from Dr. Reddy's Laboratory, India and attempt was made to standardize the protocol to prepare ^{177}Lu-labeled Reditux®.

^{177}Lu-labeled Reditux® was prepared and characterized following the same method employed for the preparation of ^{177}Lu-labeled MabThera®. Under the optimized reaction condition, ^{177}Lu-labeled Reditux® could be prepared with >95% radiochemical purity. A typical HPLC profile of ^{177}Lu-labeled Reditux® is shown in Figure 5.

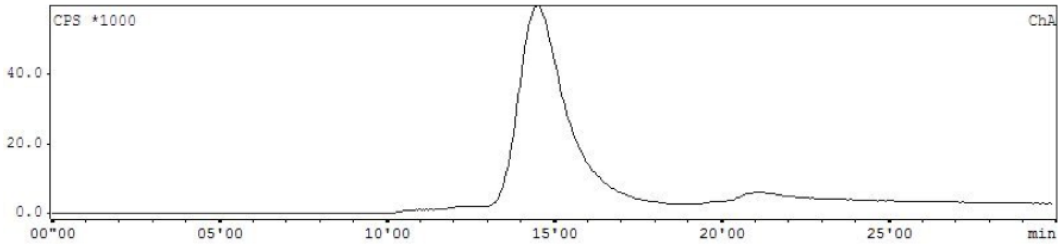


FIG. 5. Typical HPLC pattern of ^{177}Lu-labeled Reditux®.

4.4. Preparation of ^{90}Y-labeled Reditux®

For the preparation of ^{90}Y-labeled Reditux®, *p*-NCS-Bz-DOTA-Reditux® conjugate was prepared and purified following the same procedure used for the preparation of ^{177}Lu-labeled Reditux®. Subsequently, ^{90}Y-labeling of *p*-NCS-benzyl-DOTA-Reditux® conjugate was achieved by incubating 200 µg of the conjugate with $^{90}YCl_3$ (185 MBq, 5 mCi) at 37°C for 90 min using NH_4OAc buffer (pH~5.5). Characterization of ^{90}Y-labeled *p*-NCS-benzyl-DOTA- Reditux® conjugate was done by employing the HPLC technique mentioned above. A typical HPLC profile of ^{90}Y-labeled *p*-NCS-benzyl-DOTA-Reditux® is shown in Figure 6. Y-90-labeled *p*-NCS-Benzyl-

DOTA-Reditux® conjugate was prepared with >95% radiochemical purity under the optimized reaction conditions.

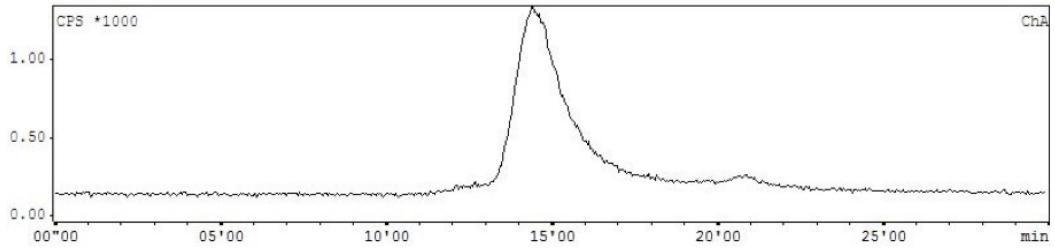


FIG. 6. Typical HPLC pattern of ^{90}Y-labeled Reditux®.

5. Development of DOTA-TATE cold kit for the preparation of ^{177}Lu-DOTA-TATE for clinical applications

In India the use of ^{177}Lu-DOTA-TATE, prepared using indigenously produced ^{177}Lu, for the treatment of patients suffering from inoperable neuroendocrine cancers over-expressing somatostatin receptors was started in 2007 and at present this treatment is provided at eight nuclear medicine centres across the country. In all these centres, ^{177}Lu-labeled DOTA-TATE is prepared at the hospital radiopharmacy based on the method developed at our centre [18]. This method enables preparation of therapeutic dose of ^{177}Lu-DOTA-TATE with highest possible specific activity at the time of administration to the patients as the composition of the ingredients is decided based on the specific activity of ^{177}Lu available during the preparation of the agent. However, the preparation requires careful adjustment of the pH of the radioactive reaction mixture prior to incubation, as the complexation yield of ^{177}Lu-DOTA-TATE achievable is highly dependent on the pH. This increases the possibility of batch failure leading to significant wastage of expensive radioactivity and peptides. To overcome this, we have recently developed a cold kit which can be utilized for the preparation of patient doses of ^{177}Lu-DOTA-TATE by a simple and single step at the hospital radiopharmacy.

DOTA-TATE cold kit was prepared by adding DOTA-TATE dissolved in supra-pure water to a solution of 0.1 M ammonium acetate (pH ~5) containing gentisic acid and subsequently freeze-drying the mixture after adjusting its pH to 5. Each kit comprises of a lyophilized mixture of 200 µg of DOTA-TATE, 80 mg of gentisic acid and 15.6 mg of ammonium acetate. Therapeutic dose of ^{177}Lu-DOTA-TATE could be prepared by adding the required volume of $^{177}LuCl_3$ with the kit material dissolved in 1 mL of water for injection and subsequently incubating the reaction mixture at 85-90°C for a period of 45 min-1 h. Quality control of the ^{177}Lu-DOTA-TATE was performed by paper chromatography (PC) and HPLC. In PC using 50% aqueous acetonitrile (v/v) as the eluting solvent, it was observed that the activity corresponding to ^{177}Lu-DOTA-TATE moved towards the solvent front (R_f = 0.5 - 0.8), while uncomplexed ^{177}Lu remained at the point of spotting (R_f = 0 - 0.1) under identical conditions. HPLC was performed by reported gradient elution technique using acetonitrile and water mixed with 0.1% trifluoro acetic acid was used as the mobile phase [19]. It was observed that upto 7.4 GBq (200 mCi) of ^{177}Lu-DOTA-TATE could be prepared in near-quantitative yield using this kit when the ^{177}Lu used is having a minimum specific activity of 740 GBq.mg^{-1} (20 Ci.mg^{-1}). The stability of ^{177}Lu-DOTA-TATE prepared using the cold kit was checked by storing the preparation at room temperature and determining its radiochemical purity at different time intervals following the quality control procedures mentioned above. It was observed that the preparation is stable upto 4 d as it retained its radiochemical purity >98% when stored at room temperature. Figure 7 (a) and (b) represent the typical HPLC patterns of ^{177}Lu-DOTA-TATE after preparation and 4 d post-preparation, respectively.

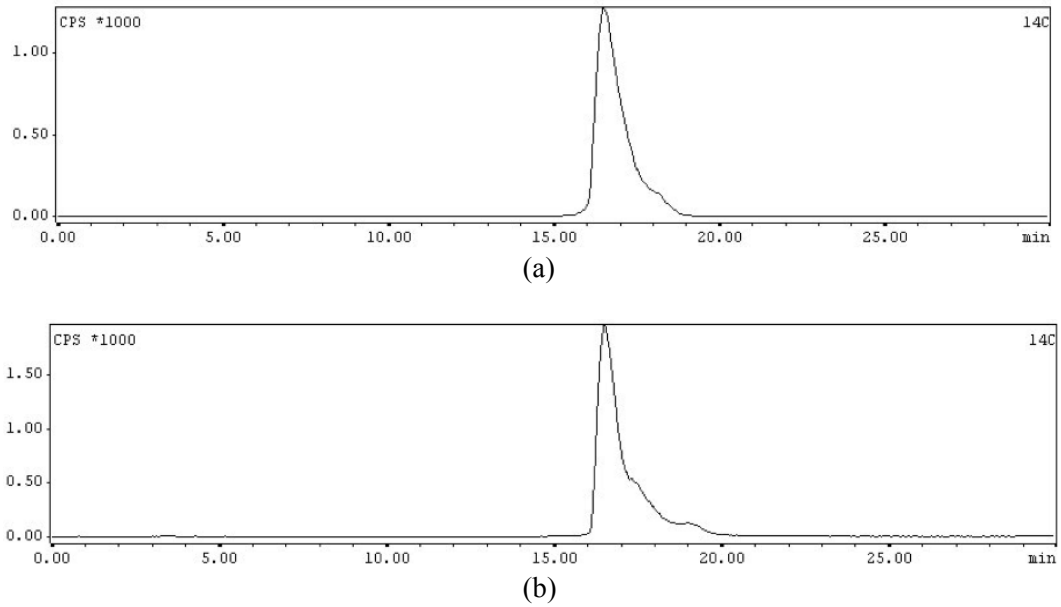


FIG. 7. Typical HPLC patterns of ^{177}Lu-DOTA-TATE complex (a) after preparation and (b) 4 d post-preparation.

6. Future plans

(1) Preparation of rituximab cold kit suitable for the preparation of patient dose of ^{177}Lu-rituximab and ^{90}Y-rituximab.
(2) Standardization of protocol for the preparation of ^{177}Lu- and ^{90}Y-labeled trastuzumab.
(3) Standardization of protocol for the preparation of ^{177}Lu- and ^{90}Y-labeled nimotuzumab.
(4) Standardization of protocol for the preparation of ^{177}Lu-labeled DOTA-Substance-P.
(5) Starting clinical studies with ^{177}Lu-DOTA-TATE prepared using the DOTA-TATE cold kit.

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ITALY (MILAN)

Conjugation of different bifunctonal chelating agents (BFC) to a monoclonal antibody

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Abstract. In the first part of this CRP we focus on the optimization of the conjugation of different bifunctional chelating agents (BFC) to a monoclonal antibody (Moab), which the IAEA has identified of scientific relevance to the member states. The implemented procedures were: (a) conjugation of the monoclonal antibodies with three different bifunctional chelating agents at different molar ratios, (b) purification of the conjugated molecules using PD-10 desalting columns, (c) ^{111}In- Radiolabeling of immunoconjugates, (d) quality control analysis to calculate the radiochemical purity and also determination of the average number of chelates per antibody, and (e) determination of stability of labelled products using an excess of DTPA.

1. Introduction

Within the scope of this CRP, ur contribution was focusing on the development of procedures for conjugation of BFC to Rituximab, radiolabeling and quality control analysis towards optimization of the radiopharmaceuticals preparation.

2.Materials

The MoAb hR3 (5 mg/mL), used has work horse in order to optimize all the procedures of conjugation and labelling of the other antibody, was provided by Cuba. The MoAb Rituximab(10 mg/mL) was provided by IAEA (10 mg/mL).

Three different bifunctional chelating agents(BFC) were used:

1. *p*-SCN-Bn-DOTA, supplied by IAEA from Macrocyclics,
2. *p*-SCN-Bn-DTPA, supplied by IAEA from Mycrocyclics,
3. 1B4M-DTPA, supplied by IAEA from Dr. M. W. Brechbiel.

Ultrapure water (Fluka) was used for all the procedures. Phosphate buffer 0.1 M (pH 8.5) was used for the Conjugation while Ammonium acetate 0.01 M (pH 7.0) for purification and radiolabeling procedures.

The precursor $^{111}InCl_3$ was purchased from Covidien. The PD-10 desalting columns were purchased from GE-Healthcare.

3. Methods

3.1. Conjugation of hR3 with BFC

The hR3 solutions (5mg/mL) were concentrated by membrane filtration using a Millipore MWCO-10000 filter for 5 min at 5300 rpm to obtain a final concentration of 10mg/mL. Then, 10 mg of the resulting hR3, were mixed with either 50- or 100-fold molar excess of BFC, previously dissolved in phosphate buffer (pH, 7.2). Then these mixtures were vortexed and adjusted to the final pH of 8.5 with NaOH (1 mol/L). The reaction mixture was incubated overnight at 4°C with gentle shaking.

3.2. Purification

The immunoconjugates were purified by size-exclusion chromatography on PD-10 desalting columns, pre-equilibrated with 25 mL of ammonium acetate 0.01 M (pH, 7.0) and then eluted with the same ammonium acetate. Four central fractions were collected (0.5 mL each), mixed together and concentrated to 5 mg/mL by membrane filtration.

3.3. ^{111}In-Radiolabeling studies

100 μCi of $^{111}InCl_3$ were added to each purified conjugates, previously dissolved in ammonium acetate buffer 0.01 M (pH 7.0), at different molar ratios and with a radiolabeling specific actvity of 0.6 mCi/mg. The solutions were incubated at 42 °C for 3 hours.

After incubation a small amount of DTPA was added in order to chelate free indium ions, and the mixture was then incubated for 15 minutes at room temperature.

3.4. Quality Control

Radiochemical purity was determined on SG-ITLC strips, and using saline as mobile phase. Radioactivity on the strip was detected with a phosphor imager (Phosphor Screen Cyclone).

3.5. Determination of the average number of chelates per antibody

The average number of chelates per antibody was determined using two different methods, which differ from the use of a conjugation mixture before and after purification and from the use of In^{3+} as a carrier.

The first method, described by Hnatowich, consists of the addition of $^{111}InCl_3$ to a small aliquot of the conjugated solution before purification. After incubation for 5 min at room temperature, ITLC analysis using saline as mobile phase was performed. Only labeled free chelates migrate whereas the labelled immunoconjugate remains at the origin. The average number of chelates groups attached per antibody molecule is calculated from the coupling efficiency. For example, assuming a 20% coupling efficiency, a 10:1 molar ratio results in an average of 2 groups per molecule [(20/100) × 10 = 2].

The second method, described by D.R. Beckford Vera, consists of the following steps:

1. addition of 0.4 nmol of purified conjugated molecules to NH_4OAc 0.5 mol/L (pH 7.0),
2. addition of 8.0 nmol of In^{3+} in a standardizied solution spiked with $^{111}InCl_3$,
3. incubation of the reaction mixture at 42°C for 3 hours,
4. addition of DTPA followed by quality control with radio-ITLC using saline as mobile phase.

The number of chelates per antibody molecule was calculated from the ratio of counts remaining at the origin to the total number of counts multiplied by the molar metal ratio (In^{3+}/mol) of the conjugate.

3.5. Stability

Stability of ^{111}In-radioimmunoconjugates was performed by incubation in DTPA excess at room temperature and samples were analyzed by ITLC after 24 and 48 hours.

4. Results

4.1. Radiochemical purity (RCP)

The radiochemical purity of the purified immunocojugates was greater than 95% using both 1:50 and 1:100 molar ratios with p-SCN-Bn-DOTA as chelating agent, whereas for the other immunoconjugates was about 70%, as shown in Table 1 below.

TABLE 1. Radiochemical purity

Radioconjugated molecules	% RCP ^{111}In-Radiolabelling
hR3 : p-SCN-Bn-DOTA = 1 : 50	98.4 %
hR3 : p-SCN-Bn-DOTA = 1 : 100	99.6 %
hR3 : p-SCN-Bn-DTPA = 1 : 50	66 %
hR3 : p-SCN-Bn-DTPA = 1 : 100	71.2 %
hR3 : 1B4M-DTPA = 1 : 50	68.1 %
hR3 : 1B4M-DTPA = 1 : 100	75 %

4.2. Conjugation

The results of conjugation are shown in Table 2, which reports the number of chelating groups (NoG) linked to the antibody as measured by the two different methods. The two methods gave different results. However, Beckford's method proved to be more accurate as it gave results closely comparable with published data (Table 3).

TABLE 2. Conjugation

Radioconjugated molecules	NoG (Hnatowich's method)	NoG (B. Vera's method)
hR3 : p-SCN-Bn-DOTA = 1 : 50	6.85	11.26
hR3 : p-SCN-Bn-DOTA = 1 : 100	9.7	12.38
hR3 : p-SCN-Bn-DTPA = 1 : 50	3.7	10.68
hR3 : p-SCN-Bn-DTPA = 1 : 100	6.8	16.02
hR3 : 1B4M-DTPA = 1 : 50	3.05	11.06
hR3 : 1B4M-DTPA = 1 : 100	8.5	12.0

TABLE 3. Comparison of results with published data

(Published results) (L/P)	NoG (Present work)
D.R. Beckford Vera (50:1)	9
D.R. Beckford Vera (100:1)	13
M. Moreau (20:1)	2.6

4.2. Stability

The results of stability measurements performed at room temperature using an excess of DTPA are shown in the Table 4 below.

TABLE 5. Stability studies

^{111}In-radiolabelled conjugates	0 hours	24 hours	48 hours
hR3 : p-SCN-Bn-DOTA = 1 : 50	99.1	97.9	94.7
hR3 : p-SCN-Bn-DTPA = 1 : 50	68.2	66.4	61.1
hR3 : 1B4M-DTPA = 1 : 50	55.0	45.2	41.4

5. Preliminary experiments with Rituximab

Preliminary experiments using Rituximab as MoAb were also performed following the same procedures. In particular, the followig steps were accomplished.

- Conjugation of Rituximab with p-SCN-Bn-DOTA as chelating agent at two different molar ratios: 1:50 and 1:100.
- Purification of the immunoconjugates by size exclusion chromatography,
- ^{111}In-Radiolabeling of purified immunoconjugates(specific activity 0.6 mCi/mg);
- Quality control analysis by ITLC.
- Determionation of the average number of chelates per antibody using Beckford method.

The results are summarized in Tables 6 and 7 below.

TABLE 6. Preliminary results on the radiolabelling of Rituximab

Radioconjugated molecole	% RCP ^{111}In-Radiolabeling

Rituximab : p-SCN-Bn-DOTA = 1 : 50	93.5
Rituximab : p-SCN-Bn-DOTA = 1 : 100	99.5

TABLE 7. Preliminary results on the conjugation of Rituximab

Immunoconjugate (L/P)	NoG (Beckford Vera's method)
Rituximab : p-SCN-Bn-DOTA = 1 : 50	18.7
Rituximab : p-SCN-Bn-DOTA = 1 : 100	19.9

6. Future Developments

Future steps will be devoted to further conduct the following studies.

- Measurement of the conjugation yield by MALDI-TOF mass spectrometry.
- Labeling of Rituximab with ^{177}Lu at increasing specific activity.
- Stability studies at 37°C in human serum.
- Evaluation of biological activity both in vitro, using antiCD-20 expressing cells, and in vivo, using lymphoma-bearing animal models (NHL).

7. Conclusions

We have (a) developed a standard conjugation procedure, (b) optimized the quality control of the immunoconjugates by checking the number of BFC per molecule of antibody, (c) performed an efficient labelling of the immunoconjugate with ^{111}In and (c) checked the stability of the labeled products using excess of a challenging agent. These basic techniques will be further utilized for further studies aimed at developing an instant kit formulation for efficient labelling immunoconjugates.

ITALY (Rome)

Centralized pre-clinical studies for ^{177}Lu-labelled antibodies and peptides

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Abstract. In the frame work of the CRP "Therapeutic radiopharmaceuticals based on ^{177}Lu and ^{90}Y labeled monoclonal antibodies and peptides: Development and preclinical evaluations" we proposed to participate as a specialized center for pre-clinical in vitro screening of kit formulations of ^{177}Lu-labelled-mAb/peptides synthesized by other laboratories participating in this IAEA coordinated project. During this year we tested in vitro biological properties of DTPA- and HYNIC-conjugated Rituximab on CD20+ve cells. Furthermore, we continued our studies on labeled superagonist rhTSH analogues testing in vitro properties and in vivo biodistribution and targeting possibilities. We developed a know-how on studying candidate radiopharmaceutical performing routinely different in vitro quality controls and binding assays. We therefore continue to offer to this CRP our skill and know-how to test the immunoreactivity and binding properties of newly developed products.

1. Introduction

The CRP in which we are participating is a co-operative effort between many laboratories with the main goal to promote the clinical application of radioimmunotherapy and peptide receptor-based radionuclide therapy for the most important cancers in developing countries. Most importantly we aim to develop an easy-to-use technology to radiolabel anti-cancer antibodies and peptides with ^{177}Lu for receptor –targeted therapy. To accomplish such an important goal, we believe that all the participant should share their best experiences in order to play their best role in this project. Our current projects are focused on the development of kit formulation to radiolabel an anti-CD20 antibody (Rituximab) and a superagonist rhTSH analogue with ^{177}Lu to treat non-Hodgkin lymphoma and non-iodine up-taking metastatic thyroid cancer, respectively. We already successfully labelled these compounds for diagnostic purposes with ^{99m}Tc using different labeling procedures. Therefore, we performed pilot experiments on 99mTc labeled DTPA- and HYNIC-Rituximab to acquire information on the binding properties of the mAb, in order to plan experiments for ^{177}Lu-labelled molecules. Furthermore, we will continue to offer our expertise, in the preliminary evaluation of kits provided by all participants, for testing all kits both in vitro and in vivo.

2. Scenario

Non-Hodgkin lymphoma (NHL) is one of the most commonly occurring hematologic malignancy and 85% of NHLs are B cell lymphomas [1]. It is common in the elderly and it is characterized by advanced stage at presentation and an indolent clinical course with a median survival of 8 to 10 years. While patients might initially respond to therapy, the disease is characterized by multiple episodes of recurrence leading ultimately to death due to refractary disease, transformation to an aggressive large B cell pathology or complications of therapy. Most patients with follicular NHL who transform to an aggressive NHL are very difficult to treat successfully. Chemiotherapy (CHT) has not a significant impact on survival, but recently, radioimmunotherapy (RIT), which uses a radiolabelled monoclonal antibody (with ^{131}I or ^{90}Y) to deliver radiation to the sites of

disease, has been extensively studied in this population with promising results. Thus RIT offers several advantages over other therapeutic modalities and in association with CHT [2]. It delivers the radiation dose specifically to the target with limited exposure of non-target issues. It is normally very well tolerated. The uptake of the radiopharmaceutical is not necessarily required by all tumour cells. Penetration of beta rays in living tissues is of several millimetres, and the therapeutic effect is obtained also in cells that do not take up the radiopharmaceutical if they are adjacent to up-taking cells by the so called cross-fire effect.

If it is possible to find a well characterized cell surface antigen over-expressed on the tumor cells, it could be possible to develop a powerful radiopharmaceutical using mAb as vehicles for radiations [3]. RIT can be an adjuvant treatment in patients where the administration of unlabelled antibody is not successful. CD20 is a preferential target for RIT of lymphomas, since it is over-expressed on malignant B cells (4). Anti-CD20 antibodies such Rituximab (Mabthera) have been developed and recently ibritumomab tiuxetan (Zevalin) labeled with ^{90}Y has been approved by FDA as a radiopharmaceutical for NHL treatment [5]. ^{90}Y is a β-emitter used for RIT, since its radiation is cytotoxic. β-particles are cytotoxic over a spherical volume with a radius that extends from a few millimeters to several centimeters. Despite initial enthusiasm, Zevalin® is now criticized by most because its poor long term efficacy, its high cost and because ^{90}Y is characterized by a systemic toxicity due to its high energy.

We therefore believe that developing an easy kit for the labeling of anti-CD20 with a more suitable isotope, such as ^{177}Lu, would provide a more suitable therapeutic tool for NHL [6]. ^{177}Lu is a short-range β emitter ($E_{\beta max}$ = 497 keV, 670μm) and this makes it ideal for treating micro-metastatic disease reducing systemic toxicity. Moreover, it emits also γ rays (208keV, 11% abundance) and could be used for dosimetric purposes. Therefore, we believe that it may have a great clinical impact the development of a kit for the rapid preparation of anti-CD20 labelled with ^{177}Lu.

In parallel, we propose to investigate also the preparation of other ^{177}Lu-labelled peptides/antibodies. In particular we initiated a project on the labelling of a newly developed TSH super-analogue that shows higher affinity and efficacy respect to the available rhTSH [7]. From our preliminary data, it appears that the ^{177}Lu-DOTA-rhTSH analogue is indeed a promising radiopharmaceutical for the treatment of non-iodine-up-taking metastases that retain the TSH receptor expression [8].

Over the years we developed an excellent know-how on mAb and peptide labelling for diagnostic and therapeutic purposes. We have a fully equipped radiochemistry analytical and semi-preparative lab with facilities for cell tissue cultures, HPLC and ITLC with detectors for gamma and beta emitting isotopes, a Ligand Tracer for automatic binding assays, autoradiography, SDS-PAGE, etc. and an animal facility equipped for animal experiments with radiopharmaceuticals, micro-SPECT, micro-PET and micro-CT. That allows us to easily screen several peptides and mAbs directed to different surface antigens expressed on tumor cells.

3. Results

Before rushing to ^{177}Lu labelling we wanted to test the biological properties of conjugated Rituximab. We compared our previous experiment on directly labelled mAb with DTPA- and HYNIC- conjugated Rituximab. Because of the lower cost and higher availability, we selected ^{99m}Tc as the isotope of choice for preliminary studies.

3.1. ^{99m}Tc-HYNIC-Rituximab

Despite the wide range of groups where HYNIC could bind on the target molecule, we have chosen to use this coligand first, because of our experience on HYNIC-conjugated molecules and the possibility to label conjugated molecules with a low cost isotope like ^{99m}Tc for preliminary studies on the biological properties of the conjugated mAb. As a first step, the bifunctional chelator was conjugated to native Rituximab in a specific modification buffer. Unbound HYNIC molecules were removed through purification with a fast and high recovery chromatographic column. The labelling was performed using tricine as a coligand and $SnCl_2$ as a reducing agent for ^{99m}Tc reduction. A titration of each reagent was performed in order to optimize the labelling

protocol. Labelling efficiency and colloid formation were measured through instant thin layer chromatography with silica gel strips as stationary phase and 0.9% NaCl solution and H_2O:EtOH:NH_3 (5:3:1) as mobile phases. Eventually, we could efficiently radiolabel HYNIC-Rituximab with a labelling efficiency of 97% and negligible amount of colloids, without the need of a further purification step. The labelled antibody was stable in both saline and human serum solution up to 24 h (37°C) and in a solution containing a physiological concentration of cysteine as well. K_d, k_{on} and k_{off} were calculated performing an in vitro binding assay using the LigandTracer. 10^7 of Raji cells (CD20+ve) were let to attach to the bottom of a tilted petri plate and the dish was placed in the rotor of the machine with 3.9 nM solution of ^{99m}Tc-HYNIC-Rituximab. When the binding reached a plateau a 1000-fold molar excess of unlabelled antibody was added for displacement and retention studies. Eventually the medium was replaced with a fresh one to measure the signal from internalized molecules. K_d was measured fitting the data with the Graph Pad software and was found to be 1.77 nM.

3.2. ^{99m}Tc-DTPA-Rituximab

We started to investigate the biological properties of DTPA conjugated Rituximab labelling the molecules with ^{99m}Tc. The mAb was conjugated with DTPA in a carbonate buffer (pH, 8.6) and the unbound chelator was removed after a purification step with a chromatographic column. The labelling was performed using $SnCl_2$ as a reducing agent and a titration was performed in order to find the best labelling conditions. The best we could reach is a labelling efficiency of 92% with marked colloid formation (23%) and a further purification step was necessary in order to achieve a high labelling efficiency (97%) with low amount of colloids (4%). The labelled mAb was stable in saline and serum solution up to 24 h (37°C) and in a solution containing a physiological concentration of cysteine as well. K_d, k_{on} and k_{off} were calculated performing an in vitro binding assay using the LigandTracer. 10^7 of Raji cells (CD20+ve) were let to attach to the bottom of a tilted petri plate and the dish was placed in the rotor of the machine with 3.9 nM solution of ^{99m}Tc-HYNIC-Rituximab. When the binding reached a plateau a 1000-fold molar excess of unlabelled antibody was added for displacement and retention studies. Eventually the medium was replaced with a fresh one to measure the signal from internalized molecules. Eventually the medium was replaced with a fresh one to measure the signal from internalized molecules. K_d was measured fitting the data with the Graph Pad software and was found to be 6 nM (native Rituximab has K_d of approximately 5 nM)

3.3. ^{99m}Tc-HYNIC-TR1401

We carried on our studies on labelled superagonist rhTSH analogue TR1401. We tested the biological properties of the labelled hormone in vitro and in vivo. LigandTracer experiments were performed on ML-1 (human thyroid carcinoma cells, TSHR+ve) cells. Briefly, 10^6 cells were grown in the lower part of a petri dish for 24 hours, then the plate was placed in the LigandTracer adding different nM concentrations of ^{99m}Tc-HYNIC-TR1401 and was allowed to rotate until the signal reached a plateau. Afterwards, an excess of unlabelled TR1401 was added for displacement studies. The data fitting was performed with the Graph Pad sotware measuring the K_d that was found to be 3 nM (the value of the native TR1401 K_d is approximately 1 nM). In vivo studies were performed in normal Balc/c mice injecting ~100 µCi in the tail vein and acquiring images with a high-resolution portable mini-gamma camera at 1, 3, 6 and 20h. The images show a high signal from the kidneys, presumably the excretion route of the radiopharmaceutical. At each time point 3 mice were sacrificed, the major organs were collected, weighted and counted in order to calculate the %ID/g. The highest value was found to be in the thyroid, whereas the signal from other organs decreased over time. Ex-vivo counts confirmed the high kidney uptake. In vivo tumor targeting experiments were performed in a mouse bearing a JP09 (TSHR+ve) tumor. Images were acquired and the target-to-background ratio was calculated with the data obtained from the .txt matrix. Highest value was reached at 6 h p.i. (3.5). The same experiment was performed in two groups of nude athymic mice. The first group was injected in the right thigh with a pellet of JP09 cells (THSR+ve) and the second group was injected with JP02 cells (TSHR-ve). After 1 hour ~100 µCi of labelled

TR1401 were injected in the tail vein and the images were acquired at 1, 3, 6 and 24 h showing a focal uptake in the right thigh of the mice injected with TSHR+ve cells. Mice injected with TSHR-ve cells showed no uptake.

3.4. Images in cats with ^{99m}Tc-HYNIC-TR1401 in collaboration with Dr Lajos Balogh

In collaboration with Hungary, we performed in vivo studies in cats in order to image the thyroid that we could not see in scans performed in mice because of its small size and relatively low uptake respect to other organs. Both cats had a tumor, which we believed to be TSHR-ve, an osteosarcoma and a head and neck tumor. In the former we were able to visualize the thyroid and the tumor was completely negative, but with surprise, the latter presented a high uptake of labelled TR1401 at the tumor site. According to data from protein bank (www.proteinatlas.org) we found out that head and nect tumors express TSHR, justifying the uptake seen in the second cat. Therefore, we plan to study more intensively our radiopharmaceutical in animals bearing different kind of tumors in order to confirm our findings.

In conclusion, HYNIC- and DTPA-conugated Rituximab has shown high affinity for the CD20 antigen and we will now proceed to study the properties of ^{177}Lu-labelled molecule repeating the same experimental protocol used for ^{99m}Tc-HYNIC- and ^{99m}Tc-DTPA-Rituximab. At the same time, we renew our proposal to help in this CRP by offering our skill and know-how to test the immunoreactivity and binding properties of other products developed by collaborating laboratories. We remind that we can also perform biodistribution and targeting studies in MICO-luc autofluorescent mice. Finally, in collaboration with Dr Balogh, we will continue our studies on labeled superagonist rhTSH analogue with ^{99m}Tc and ^{177}Lu for diagnostic and therapeutic applications in patients with non-iodine up-taking differentiated thyroid cancer

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REPUBLIC OF MACEDONIA

Establishment and standardization of a technology for the production of ready-to-use cold kit formulations for labelling DOTA-Rituximab and peptide-based conjugates with Lu-177 and Y-90

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1. Introduction

The purpose of this investigation was to introduce a technology for the production of ready-to-use cold kit formulations for labeling DOTA-Rituximab and peptide-based (Substance P) conjugates with Lu-177 and Y-90, and to firmly establish and standardize the resulting methods for synthesis and conjugation.
Concomitantly, the biological properties and pharmacokinetic behaviour of radiolabelled ^{177}Lu-DOTA-Rituximab and ^{177}Lu-Substance P were investigated using experimental systems comprising both isolated cell cultures and animal models.

2. Labelling of Substance P

Endogenous Substance P [1] is an undeca- neuropeptide that functions as a neurotransmitter and neuromodulator. It belongs to the tachykinin neuropeptide family. Substance P and its closely related analogue neurokinin A (NKA) are produced from a polyprotein precursor after differential splicing of the preprotachykinin A gene.

Owing to the ideal nuclear properties and richer chemistry of Tc-99m, and of its theranostic counterpart Re-188, it was first considered convenient to start a preliminary labelling study using these radionuclides with the purpose to evaluate the bioditribution behaviour of the radiolabelled peptide before investigating a peptide derivative suitable for binding to Lu-177. Thus, a modified peptide sequence, suitable for labelling Substance P with Tc-99m and Re-188, was synthesized and its biological characteristics examined by in vivo biodistribution studies in normal mice.

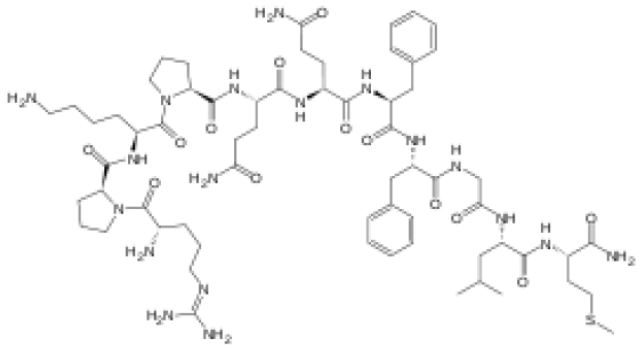


FIG.1. Chemical structure of the peptide Substance P (aminoacid sequence: Arg Pro Lys Pro Gln Gln Phe Phe Gly Leu Met).

2.1. Syntesys of modified Substance P

A peptide with the sequence Cys-Cys-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met (Cys-Cys-SP), where the groupment of two terminal cysteine residues served as chelating site for the metal, was synthesized for ^{99m}Tc-labelling. Additional ligands utilized for the preparation of the corresponding ^{99m}Tc-complex were tris-cyanoethyl phosphine [PCN = $P(CH_2CH_2CN)_3$] as ancillary ligand, succinic dihydrazide [SDH = H_2N-NH-C(=O)-CH_2-CH_2-C(=O)-NH-NH_2] as donor of nitrogen atoms to form the ^{99m}Tc-nitrido core, and tin chloride as reducing agent. Similarly, a peptide characterized by the sequence IsoCys-Cys-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met (IsoCys-Cys-SP), where IsoCys represents isocysteine, was obtained for labelling with Re-188, while keeping the other additional ingredients exactly as employed with Tc-99m [2].

2.2. Preparation of the conjugate complex [^{99m}Tc(N)(Cys-Cys-SP)PCN]

A solution containing 0.9 mL of Na[$^{99m}TcO_4$] (100MBq) containing 5.0 mg of SDH and 0.10 mg of $SnCl_2$ was added to a vial. The mixture was kept at room temperature for 15 min. To the resulting mixture, 0.5-0.05 mg of Cys-Cys-SP (dissolved in 0.5 mL of saline) and 0. 5 mg of PCN (dissolved in 0.5 mL of saline containing 2.0 mg of gamma-hydroxypropylcyclodextrin) were simultaneously added. The vial was heated at 100° C for 30 minutes. The radiochemical yield was determined by HPLC chromatography. The final pH was 6-7. A pictorial illustration of the molecular stucture of the resulting Tc-99m peptide conjugate complex is reported below.

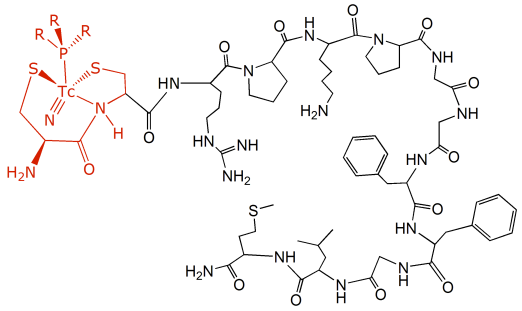


FIG. 2. Drawing of the molecular structure of the ^{99m}Tc-Substance P conjugate complex.

2.3. HPLC chromatography

Radiochemical purity (RCP) was checked by HPLC (Beckman System) using the following chromatographic conditions. Column, Zorbax 300SB-C18, 300Anst., 5 micron (4.6 × 250 mm) with a guard column; detector, UV and radiometric, mobile phase, A (0.1 % TFA in water), B (0.1 % TFA in CH_3CN); flow rate, 1 ml/min; gradient, 0-30 min, %B 0-100, 30-32 min, %B, 100, 32-34 min, %B, 100-0. Retention time was 15.8 min.

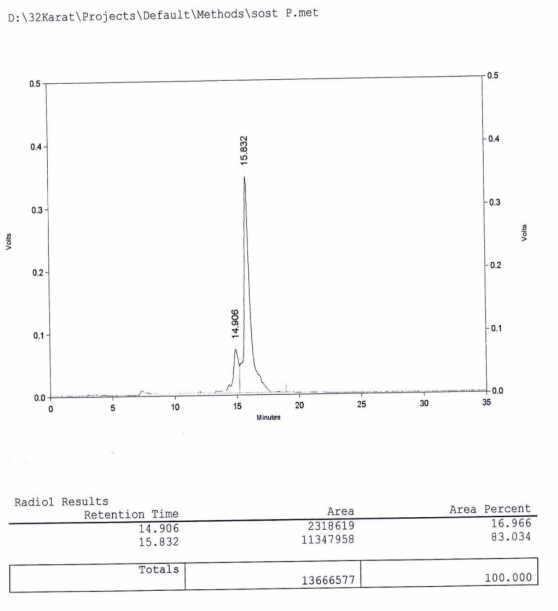


FIG. 3. HPLC chromatogram of [^{99m}Tc(N)(Cys-Cys-SP)PCN].

2.3. Stability

To evaluate inertness of the ^{99m}Tc-peptide conjugate towards transchelation by potential endogenous ligands, it was incubated in solutions containing increasing amounts of cysteine and glutathione. Determination of radiochemical purity (RCP) after incubation revealed that the radiocompound was highly resistant to substitution by competing ligands. Similarly, incubation was performed to evaluate the stability of the ^{99m}Tc-complex in both mouse, rat and human serum. It was found that the complex was not stable in rat serum and started to decompose after 30 min of incubation. Surprisingly, this decomposition was not observed in mouse serum and slowed down in human serum starting only after 60 min of incubation. This result provides another example of the strong dependence of the biological properties of some radiolabelled molecule on the specific animal species.

2.4. Preparation of the conjugate complex [^{188}Re(N)(IsoCys-Cys-SP)PCN]

A solution containing 1.5 mL of Na[$^{188}ReO_4$] (100 MBq) containing 5.0 mg of SDH and 0.10 mg of $SnCl_2$, 1.5 mg of ascorbic acid, 28 mg of sodium oxalate and 0.1 mL of glacial acetic acid, was added to a vial. The mixture was kept at room temperature for 15 min. To the resulting mixture, 0.5-0.05 mg of IsoCys-Cys-SP (dissolved in 0.2 mL of saline) and 0. 5 mg of PCN (dissolved in 0.3 mL of saline containing 2.0 mg of gamma-hydroxypropylcyclodextrin) were simultaneously added. The vial was heated at 100° C for 1 hour. The radiochemical yield was determined by HPLC chromatography using the same conditions as detailed for the Tc-99m preparation. The final pH was 4.5. Retention time was 16.1 min.

3. Biodistribution studies with a YAP(S)PET small-animal tomograph

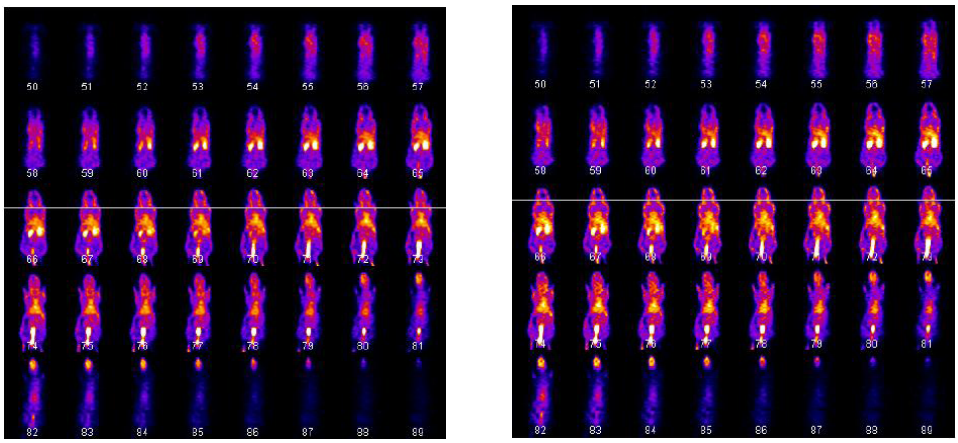
Biodistribution of the Tc-99m conjugate complex with Substance P was carried out in a normal mouse using a multimodality YAP(S)PET-CT small-animal scanner [3]. Though this animal model is the most commonly employed for this type of imaging, in the present study, it was also the most advantageous since the ^{99m}Tc-Substance P conjugate exhibited high stability in this animal species.

3.1. SPECT data acquisition

SPECT-CT studies were performed on anesthetized normal mouse (~ 25 g) injected with 75 MBq (2 mCi) of ^{99m}Tc-Substance P to study the tracer whole-body distribution. The mouse was scanned starting from 20 min after injection . The SPECT acquisition consisted of 3 bed position, 3 cm apart, 20-min acquisition time for each position, 128 views over 360°. The second acquisition starts at 90 min after the injection with the same modalities. The energy window was 140-250 keV and the images were reconstructed using the iterative EM-ML algorithm including the collimator response.

3.2. X-ray data acquisition

The radiographic images have been acquired using a digital X-ray imaging system integrated with the YAP(S)PET scanner. The acquisition parameters for X-ray projections were: X-ray-tube voltage 35 kV, anode current 1 mA, exposure 1 s, 8 views over 360° and magnification factor 1.2. Each final image was obtained by subtracting dark noise contributions and performing flat field corrections.



(a) (b)

FIG. 4. SPECT mouse coronal sections at (a) 20 min and (b) 90 min post injection of ^{99m}Tc-Substance P.

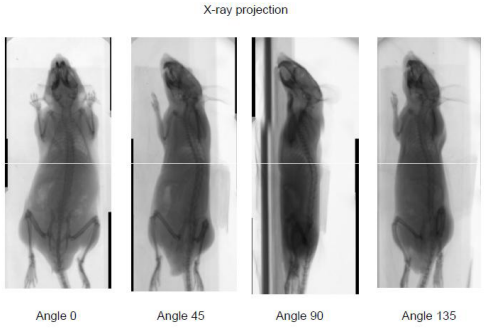


FIG.5. X-ray mouse transparent whole-body images.

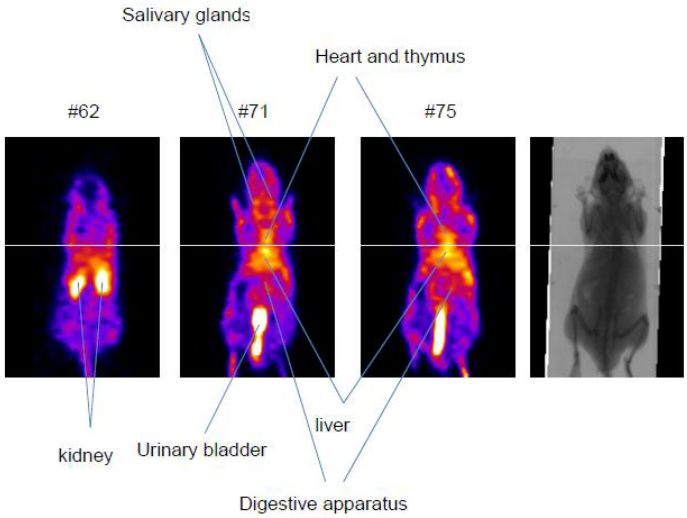


FIG. 6. Comparison of non-superimposed SPECT and CT (transparent) mouse coronal sections showing uptake in target and not-target organs for ^{99m}Tc-labelled Substance-P.

3.3. Results

Imaging studies with small-animal scanners are particularly useful for obtaining a whole in vivo picture of the pharmacokinetic behavior of a tracer. SPECT whole-body images at different acquisition times showed that the radiocompound was eliminated through the kidneys into urine as expected for a peptide-based compound. Liver uptake was almost negligible and yielded also some uptake into the gatsrointenstinal tract. Signature of the specific receptor interaction characteristic of the peptide Substance P was demonstrated by the high uptake in thymus, which is a receptor-rich organ for this peptide. At earlier times post injection, prolonged persistence of activity into the blood stream was observed, which allowed imaging of the cardiac area. Although, blood washout occurred at later times points, this did not prevent the clear visualization of the thymus gland, thus indicating high tracer uptake in this organ according to its high receptor density. This observation was also supported by ex-vivo experiments (not shown here) that measured uptaked activity after removal of the gland. It is worthy to note that the analogous Re-188 peptidic complex showed almost identical biosditribution behaviour (not reported here) as evidenced on images collected by exploiting the gamma emission associated with the beta decay of this radionuclide. This result further supports the principle that a couple of Tc-99m and Re-188 radiocompounds having exactly the same ligand arrangement and molecular structure always constitutes a true example of theranostic (diagnostic/therapeutic) pair.

4. Studies on the development of a suitable freeze-drying protocol for antibodies

The other part of our work was aimed at establishing a convenient procedure for freeze-drying antibody's solutions without compromising immunoreactivity and ensuring sterile and pyrogen-free conditions. This preliminary achievement is essential to support the purpose of obtaining suitable ready-to-use instant kit formulation for labeling antibodies with Lu-177 and Y-90.

The procedure for freeze-drying should provide a stable kit formulation giving identical labelling results as the freshly prepared liquid formulation while preserving immunoreactivity of the conjugated antibody. This condition has to be tested both before conjugation of the antibody to some chelating ligand for the metal, and after conjugation. For this reason, the most important parameters to be primarily evaluated are the freeze-drying

time required in each phase, namely (a) prefreezing, (b) primary drying and (c) secondary drying, and freezing temperature. Successively, the following properties have to be tested:

- Appearance,
- Specific surface area,
- Sublimation rate,
- Residual moisture and recon time,
- Physical state of protein and excipients in the lyophilized cake,
- Crystallinity,
- Protein aggregation, changes in conformation and bioactivity,
- Stability over time.

4.1. Representative freeze-drying procedure for proteins

As an example of freeze-drying protocol for proteins, the following standard procedure was investigated as applied to human serum albumin (HSA), immunoglobulins (IgGs), and antibodies (Mabthera). Specific conditions and parameters were as detailed below.

- Volumne of solution: 1mL
- Filled into 2-mL glass vial (fill depth = 0.75 cm)
- Freeze drier model LABCONCO

4.1.1. Procedure

- Ramp from room temperature to - 45 ^{0}C , (ramp rate 1 ^{0}C/min).
- Hold for 2 hours, then ramp to -20 ^{0}C (1^{0}C/min).
- Hold for 1 hours, then return to -45 ^{0}C .
- Maintain shelf temperature for 2 hours.
- Primary drying conducted at chamber pressure (Pc) of 57 mTorr and shelf temperature of -25 ^{0}C and +25 ^{0}C.
- Keep chamber pressure (Pc) was constant for primary and secondary drying.
- Prymary drying was carried out 4 min for HSA and 3 min for IgG and mAb.
- Perform secondary drying.
- Shelf temperature at 40 ^{0}C for 10 hours (increase interval 15-20 hours, ramp rate 1 ^{0}C/min)

4.1.2. Results

The stability of the resulting freeze-dried formulations have been measured at different times (6 months for IgG, 10 months for HSA and 3 months for Mabthera) and Figure 5 reports the radiochemical purity after labeling of lyophilized HSA with I-125.

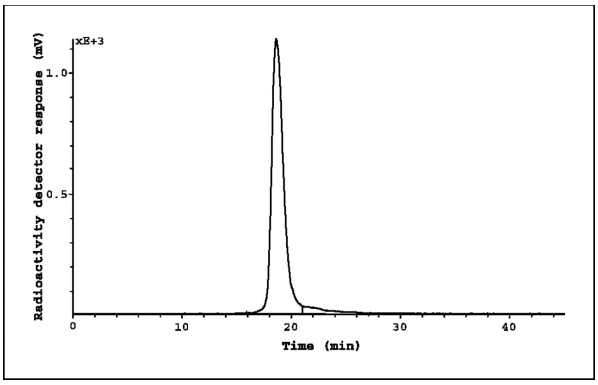


FIG.7. Freeze-dried HSA radiolabelled with I-125 after 10-month storage as checked by radiometric HPLC.

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POLAND

Polish experience in the development and preclinical evaluation of therapeutic radiopharmaceuticals based on Lu-177 and Y-90 labeled monoclonal antibodies and peptides

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Abstract. DTPA- or DOTA-chelated antibodies have been proved to be efficient in radioimmunotherapy of cancer after radiolabelling with beta-emitting radiometals. The standardization of methodology for conjugation of suitable chelators to monoclonal antibodies and their further pharmaceutical development were planned in the framework of IAEA coordinated research project on the development of radiopharmaceuticals labeled with ^{90}Y or ^{177}Lu. Therefore, our study was focused on the development of dry kit based on DOTA-anti-CD20 for ^{177}Lu labeling as potential radiopharmaceutical for radionuclide therapy.

1. Introduction

The development of new target-specific radiopharmaceuticals for diagnosis and therapy of different disease remains a challenge for the scientific community. Monoclonal antibodies (mAb) are attractive molecules in the field of molecular imaging agent development, partly because of the development of personalized medicine. This field of research is in constant evolution in order to develop and improve labeling techniques suitable to antibodies half-life for both preclinical biodistribution studies and further possible applications in diagnosis and treatments. Rituximab (trade names Rituxan and MabThera) is a chimeric monoclonal antibody against the protein CD20, which is primarily found on the surface of B cells. Rituximab destroys B cells, and is therefore used to treat diseases which are characterized by excessive numbers of B cells, overactive B cells, or dysfunctional B cells. This includes many lymphomas, leukemias, transplant rejection, and some autoimmune disorders.

Lu-177 ($T_{1/2}$ = 6.7 days, E_γ = 0 .208 MeV, $E_{\beta max}$ = 0.497 MeV, max tissue penetration = 2.0 mm) is being strongly considered for RIT mainly because of its availability, the convenient energy of its beta particles and the ability to form stable complexes with macrocyclic and acyclic ligand. Its half-life is appropriate for preparation, transportation and successful delivery of therapeutic doses to the tumor by radioimmunoconjugates (mAb).

In the first 18 months of the CRP we focused on the conjugation of Rituximab with macrocyclic bifunctional chelating agents, the optimization of ^{177}Lu labeling of the conjugates DOTA(SCN)-Rituximab and DOTA(NHS)-Rituximab and on the freeze-dried kit formulation for both conjugates.

2. Optimization of conditions for antibody conjugation and labelling of the DOTA-conjugates with Lu-177

2.1. Materials

Chimeric anti-CD20 monoclonal antibody Rituximab (MabThera 100 mg/10 ml) was provided by Roche. Lu-177 c.a. (specific activity above 555 GBq/mg Lu) as chloride solution, was provided by National Centre for Nuclear Research, Radioisotope Centre POLATOM. Isothiocyanate-benzyl-DOTA (p-SCN-BZ-DOTA) and DOTA-(N-hydroxysuccinimide ester) were purchased from Macrocyclics. Ultrafilter: Amicon® Ultra- 2ml, 10K: MWCO 10 000; Millipore. PD-10 column for purification of DOTA-mAb: PD-10 Desalting Column, GE Healthcare. Kit for determination of protein concentration: BCA™ Protein Assay Kit, Pierce, Il, USA, no. 23225.

To reduce metal contamination, all the solutions used in conjugation and radiolabeling reactions were passed through a Chelex 100 column in a following procedure: 5 g of Chelex 100 was dissolved in 15 ml of deionized water and transferre slowly to Agilent Bond Elut Reservoir column then the column washed at the flow rate of around 1 ml/min with 5 ml 0.1M HCl followed by deionized water until pH of effluent was ~ 7.0. Such prepared column could be used for purification of around 20 ml of buffer solution. Columns for filling with Chelex resin: Agilent Bond Elut Reservoir, 6 ml capacity, Agilent Technologies.

2.2. Antibody conjugation

Two different chelators p-SCN-DOTA and DOTA-NHS-ester were conjugated to anti-CD20 antibody with appropriate molar excess of chelator over antibody (mAb:DOTA) respectively of 1:5; 1:10 for p-SCN-DOTA and 1:33, 1:75, 1:100 for DOTA-NHS-ester.

2.2.1. Preparation of antibody solution for conjugation

The solution of MabThera containing 20 mg of antibody was purified by double filtration using Amicon Ultra-2 ml centrifuge filter (30 min, 5000 rpm). The final volume was reduced to < 0.5 ml (40 mg/ml).

2.2.2. Conjugation of anti-CD-20 with p-SCN-Bn-DOTA

The concentrated mAb solution was incubated with p-SCN-DOTA in 250 µl of 0.2 M carbonate buffer, pH 9.5 at 37 ^{0}C for 90 min in molar ratios of 1:5 or 1:10.

2.2.3. Conjugation of anti-CD-20 with DOTA-NHS-ester

The concentrated mAb solution was incubated with 1ml of 40-mM DTPA at 4 ^{0}C for 40 min following by purification on PD-10 using 0.1-M sodium phosphate as a mobile phase. Then the purified mAb was incubated with 1:33, 1:75 and 1:100 excess of DOTA-NHS-ester at 4 ^{0}C for 48 h.

2.3. Purification of DOTA-Rituximab conjugates

To remove the non-reacted chelators the immunoconjugates were purified by size-exclusion chromatography on PD-10 desalting columns pre-conditioned with 0.25-M ammonium acetate, pH 7.0. Fractions of 0.5 ml were collected. Concentration of antibody in the collected eluates was determined by BCA method. Number of coupled DOTA molecules was determined using Pb:Arsenazo III reaction.

2.4. Determination of antibody concentration

Antibody concentration in the eluates obtained in 2.2.1. and 2.3. was determined by BCA method using the kit provided by Pierce and according to the instructions delivered with the kit. When using the microplate procedure the samples from the collected fractions should be used after 5-fold dilution in 0.25 M ammonium acetate, pH 7.0. For the fractions containing the highest concentration of antibody (usually fractions 6-9) the purity of obtained conjugate was assessed by HPLC. Fractions having the purity confirmed by HPLC were collected in one vial.

2.5. Quality control

The radiochemical purity of the labeled conjugates and their stability were determined using SE-HPLC and TLC.

2.5.1. SE-HPLC

Column, BioSep-SEC-s2000 (300 × 7.5 mm (Phenomenex); flow rate, 1ml/min; isocratic elution, eluent 0.1-M phosphate buffer, pH 5.8, UV detection at 220 and 280 nm, analysis time ca. 20 min, sample volume, 20 µl.

In this system the retention time for DOTA-Rituximab conjugates was ca. 6.6 min.

2.5.2. TLC

TLC- Silica Gel (ITLC-SG) strips (Pall Life Science, USA) using 0.1 M sodium acetate buffer pH 5.0 as developing solvent.

2.6. Determination of number of conjugated DOTA molecules

Color reaction is based on the formation of Arsenazo III complexes with metal cations. The method was described in: E. Dadachowa, L. L. Chapell and M. W. Brechbiel, Nucl. Med. Biol, Vol 26, 977-982, 1999.

2.6.1. Procedure

Solution of Arsenazo III (AAIII) – Pb: 5-µM Pb(II), 10-µM AAIII in 0.25-M ammonium acetate pH 7.

Tested sample: to 980 µl of AAIII-Pb solution add 20 µl of 5-fold diluted conjugate. After 10-min incubation, measure the absorbance at 656 nm in the UV-VIS spectrophotometer against 0.25-M ammonium acetate.

Blank sample: to 980µl of AAIII-Pb solution add 20µl of 0.25M ammonium acetate pH7. After 10 min incubation measure the absorbance at 656 nm in the UV-VIS spectrophotometer against 025M ammonium acetate.

The number of conjugated DOTA molecules was calculated according to Lambert-Beer equation,

$$C_{DOTA} = \frac{(A_P - A_0) \cdot 5 \cdot 1000 / 20}{\in},$$

where A_0 = absorbance of blank, A_P = absorbance of tested sample and ∈ = absorbance coefficient given by the relation,

$$\in = \frac{A_0}{b \cdot c},$$

where ∈ = absorbance coefficient, b = length of optical pathway and c = concentration of Pb in the AAIII-Pb solution.

It is highly recommended to assay the number of DOTA molecules from at least 3 independent samples.

2.7. Storage

The purified conjugates of DOTA(SCN)-Rituximab and DOTA(NHS)-Rituximab were divided into portions containing 1-1.5 mg mAb in about 100-150µl and were stored at -20 ^{0}C.

The immunoconjugate of DOTA(NHS)-Rituximab (1 ml, 2.4 mg/ml) was also prepared in the kit form by lyophilization with 5 mg of mannitol and was stored at 2-8 ^{0}C.

2.8. ^{177}Lu radiolabelling of DOTA-Rituximab

To around 1.0 mg of DOTA-Rituximab in 100 µl volume 50 µl of 0.5 M ammonium acetate pH 7.0 and [^{177}Lu]$LuCl_3$ (LutaPol) with radioactivity about of 250-2200 MBq were added. Afterwards, the reaction mixture was incubated at 38^{0}C from 30 min to 2 h. An aliquot of 10-20 µl was then used for the analysis by HPLC as described below.

2.8.1. HPLC analysis

To around 20 µl of radiolabelled conjugate 10 µl of 10 mM DTPA solution was added in order to bind non-reacted ^{177}Lu. HPLC analysis was performed 5 min after DTPA addition with method described in 2.5., and using UV detection at 220 nm, 280 nm and radiometric detection. ^{177}Lu-DOTA-Rituximab, retention time ~ 6.6 min, ^{177}Lu in the DTPA complex, retention time ~ 9.7min.

2.9. Stability studies

The stabilities of radiolabelled conjugates were evaluated after storage preparations at room temperature and after incubation in human serum albumin (HSA). SE-HPLC method as described above was used for radiochemical purity determination.

2. Results

The conjugation of DOTA(SCN) to mAb was performed using five and tenfold excess of p-SCN-Bn-DOTA, resulting in a DOTA-rituximab conjugate with an average ratio of 3 and 5 DOTA molecules per antibody respectively, see Table 1. The table presents also the results of stability of ^{177}Lu-DOTA(SCN)-mAb.

TABLE 1. Stability of ^{177}Lu-DOTA(SCN)-Rituximab

Molar excess of chelator DOTA:Ab	Average numer of conjugated DOTA(SCN)	Radiochemical purity, %		
		0.5 h	2.0 h	24.0 h
5:1 (n=2)	3:1	89.3±9.6	87.1±10.6	79.4±9.2
10:1 (n=3)	5:1	96.6±5.0	92.2±6.1	90.9±5.1

An example of HPLC profile of ^{177}Lu-DOTA(SCN)-Rituximab with UV and radiometric detection is presented in Fig. 1 and 2.

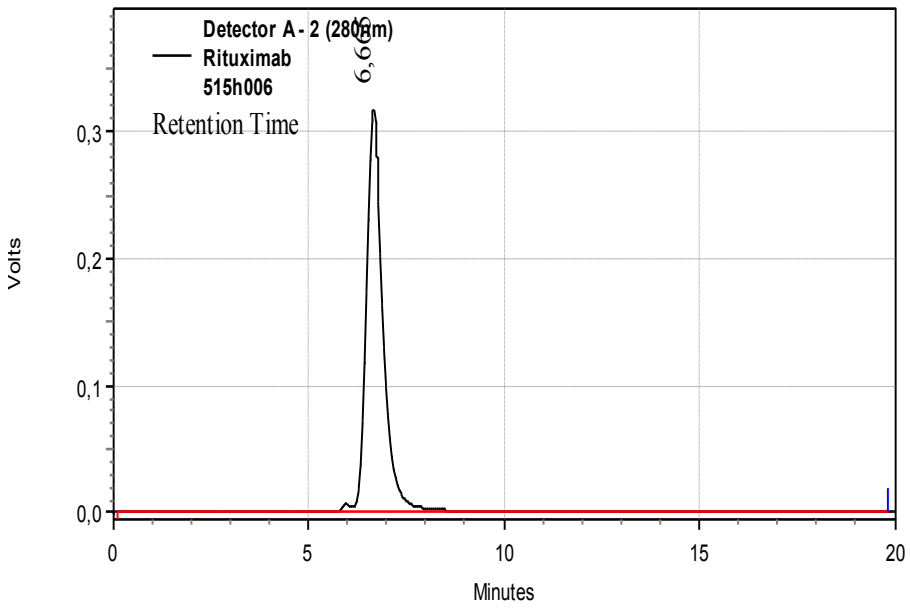

FIG. 1. HPLC-UV profile of the DOTA(SCN)-Rituximab.

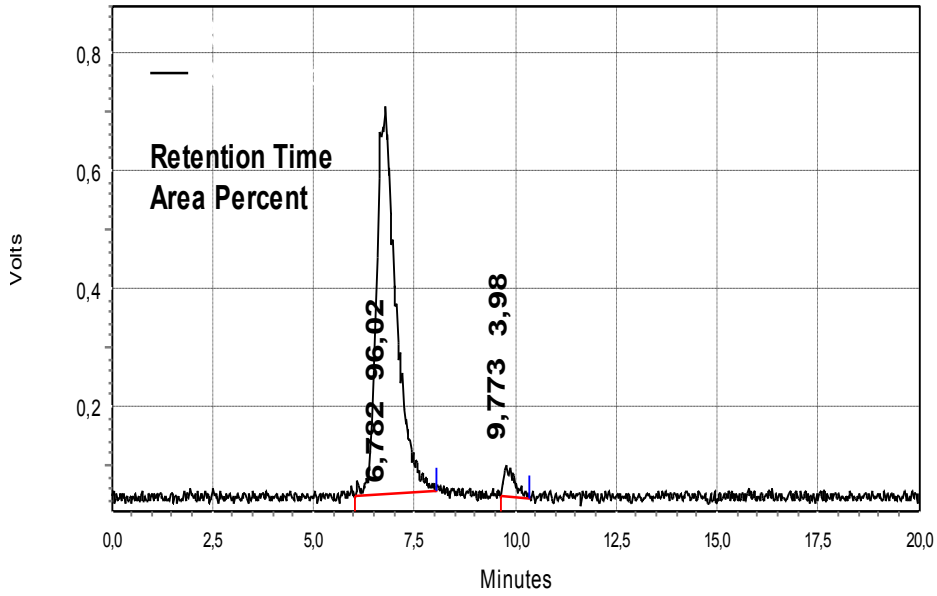

FIG. 2. HPLC-radiometric profile of ^{177}Lu-DOTA(SCN)-Rituximab.

Fig. 3 presents the results of ^{177}Lu-DOTA(SCN)-Rituximab in 1% HSA indicating that the radiochemical purity of ^{177}Lu-DOTA(SCN)-Rituximab was higher than 90% up to 24 hours.

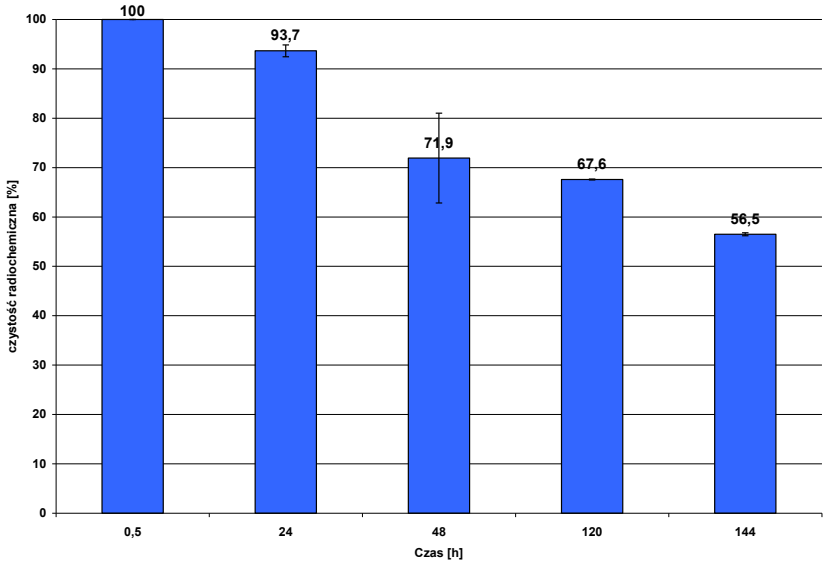

FIG. 3. Stability of ^{177}Lu-DOTA(SCN)-Rituximab in HSA.

The conjugation of DOTA(NHS)-ester to rituximab was performed using 33, 75 and 100-fold excess of DOTA-NHS-ester, resulting in a DOTA-rituximab conjugate with an average ratio of 9 and 10 DOTA molecules per antibody respectively, see Table 2.

TABLE 2. Stability of ^{177}Lu-DOTA-Rituximab at RT

Initially molar ratio DOTA:Ab	Average numer of conjugated DOTA	Radiochemical purity, %		
		0.5 h	2.0 h	24.0 h
33:1 (n=1)	7:1	19.0	20.5	-
75:1 (n=2)	9:1	92.7±3	87.5±1.5	-
100:1 (n=3)	10:1	98.1±0.4	97.6±1.1	95.6±3.0

Representative HPLC profiles of ^{177}Lu-DOTA-Rituximab with UV and radiometric detection are presented in Figs. 4 and 5.

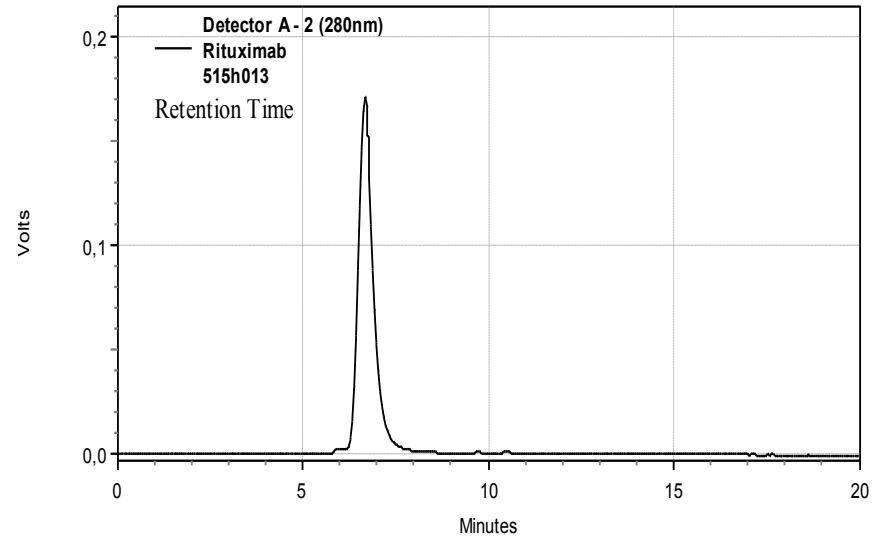

FIG. 4. HPLC-UV profile of the DOTA-Rituximab.

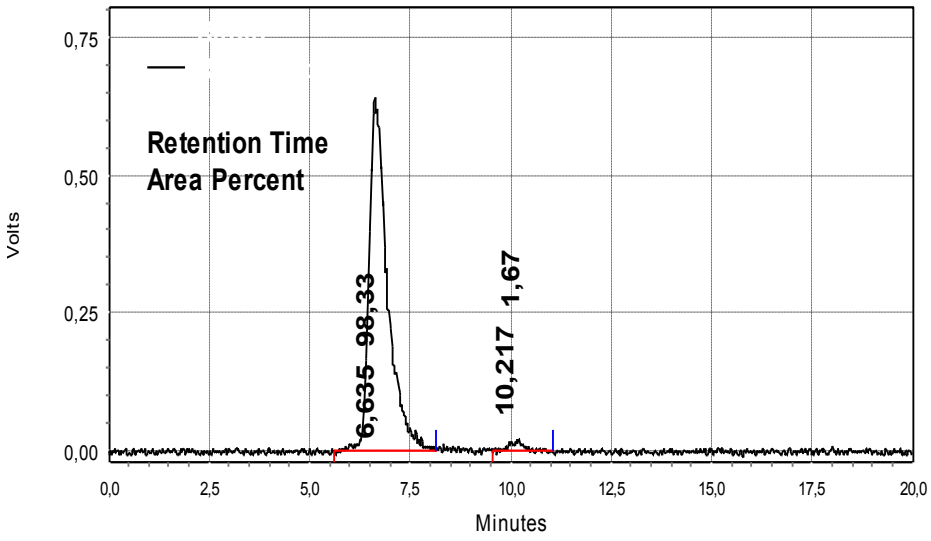

FIG. 5. HPLC-radiometric profile of ^{177}Lu-DOTA-Rituximab.

The stability of ^{177}Lu-DOTA-Rituximab prepared in the wet labelling procedure and using the freeze–dried conjugate are compared in Fig. 6. No significant differences were observed between the two preparations stability.

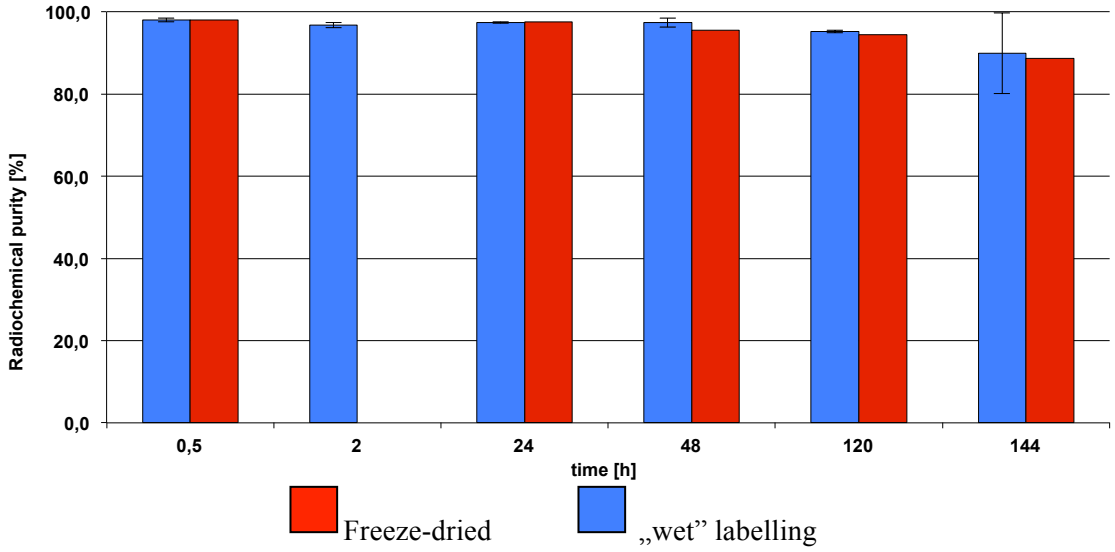

FIG. 6. Comparison of stability of ^{177}Lu-DOTA(NHS)-Rituximab using freeze-dried conjugate and the conjugate stored in 0.5 M ammonium acetate buffer, pH 7.0 at -20^{0}C.

3. Conclusions on antibodies

The aim of our study was to prepare the DOTA- conjugates with mAb and to prepare such conjugate in the form of dry kit which would allow rapid and reporducible labelling of the DOTA-mAb.

Optimized conditions of radiolabelling at pH 7.0 (1.2 mg of DOTA(SCN)-Rituximab, ^{177}LuCl3 up to 1.4 GBq in 50 µl of 0.5 M ammonium acetate pH 7.0, 2 hours incubation at 38^{0}C) resulted in ^{177}Lu-DOTA-Rituximab with specific activity of 1.1 GBq/mg (158 GBq/µmol). The labeling yield was found to be 92-94% at 30 min and increased to > 97 % at 2 hours incubation. High radiochemical yields above 95% were obtained for mAb conjugated at 1:10 molar ratio. ^{177}Lu-DOTA(SCN)-Rituximab was stable up to 24 h in HSA at RT.

High radiochemical yield above 98%were obtained for DOTA-NHS conjugated mAb at 1:100 molar ratio. Freeze-dried conjugate containing 2.4 mg mAb, 5 mg mannitol, 3.32 mg sodium acetate was prepared. Radiolabelled conjugates (both wet and freeze-dried conjugates) were stable up to 120 h at RT.

4. ^{177}Lu-DOTA-Substance P

Gliomas, WHO grades II-IV, have been shown to consistently overexpress the transmembrane neurokinin type I receptor (NK-1). NK-1 receptors have also been detected on tumour cells infiltrating the intra- and peritumoral vasculature. The physiological ligand for NK-1 receptor is Substance P. This peptide can be labelled with various radionuclides for diagnostic and therapeutic applications using the chelators. Recently the successful appliaction of targeted alpha-radionuclide therapy of functionally critically located gliomas with ^{213}Bi-DOTA-[Thi^{8}, Met(O$_2$)^{11}]-substance P has been published by Cordier et al., 2010 [6]. These authors used ^{111}In-DOTA-[Thi^{8}, Met(O$_2$)^{11}]-substance P for evaluation of receptor expression and dose distribution with 3-D data set (SPECT and low-dose CT) immediately after injection and at later time points. The aim of our work was to use ^{68}Ga DOTA-[Thi^{8}, Met(O$_2$)^{11}]-substance P PET imaging for the possible pre-therapeutic assessment of receptor expression and therapeutic dose distribution. The first dose of ^{213}Bi/^{68}Ga- DOTA-[Thi^{8}, Met(O$_2$)^{11}]-substance P

was administered to the patient. Therapeutic application of ^{177}Lu-DOTA-[Thi^{8}, Met(O$_2$)^{11}]-substance P is planned.

5. Methods

5.1. Labelling of DOTA-[Thi^{8}, Met(O$_2$)^{11}]-Substance P

5.1.1. Labelling of DOTA-[Thi^{8}, Met(O$_2$)^{11}]-substance P with ^{177}Lu

For radiolabelling, to 1.0 mL of a 5% ascorbic acid solution (pH = 4.5) 50 μg DOTA-[Thi^{8},Met(O$_2$)^{11}]-substance P (piChem, Austria) and 1.85 GBq of ^{177}LuCl$_3$ (S.A. > 555 GBq/mg Lu) were added. The reaction mixture was incubated at 95°C for 25 min.

5.1.2. Labelling of DOTA-[Thi^{8}, Met(O$_2$)^{11}]-substance P with ^{68}Ga

For radiolabelling, first 3 mL of eluate of the ^{68}Ge/^{68}Ga generator (iThembaLABS) with radioactivity of 350 – 780 MBq of ^{68}Ga was added of 100 μg DOTA-[Thi^{8},Met(O$_2$)^{11}]-substance P dissolved in 2.4 mL 1.25 M AcONa, giving final pH of about 3.7. Incubation was carried out at 95^{o}C for 15 min using heating oven.

5.2. Quality control

For determination radiochemical purity of the labelled preparations several methods were used.

5.2.1. HPLC

A solvent module (Shimadzu LC20A) with UV detector and online γ-ray detector (Raytest, Gabi) was used for HPLC analysis. Reversed phase HPLC was performed with JupiterProteo 4u 90A column (4.6 x 150 mm, Phenomenex) at the gradient or isocratic condition. The flow rate was 0.6 ml/min and UV detection at 220 nm. Solvent system for gradient elution: solvent A – 0.1 % TFA in Acetonitrile, solvent B – 0.1 % TFA in water was used. Gradient: 0-9 min. from 18% to 60% A; 9-13 min. 60 0% A; 13-18 min. from 60 to 18% A, 18-23 min 18 % A; 20-25 min 95% B. Before injection to the 25 μl of tested sample the 25 ml solution containing 1.0 mg/ml DTPA were added to bind free radionuclide. In these conditions the retention time of ^{177}Lu-DTPA is 3.0 min, and that of ^{177}Lu and ^{68}Ga- DOTA-[Thi^{8},Met(O$_2$)^{11}]-substance P is 9.3 min. For isocratic elution solution containing 25% of 0.1 % TFA in Acetonitrile and 75% of 0.1 % TFA in water was used. In these case the retention time of ^{177}Lu-DTPA is 2.5 min, and that of ^{177}Lu and ^{68}Ga- DOTA-[Thi^{8},Met(O$_2$)^{11}]-substance P is 5.9 min.

5.2.2. TLC

For determination of the content of the unbound ^{177}Lu or ^{68}Ga the thin layer chromatography analysis was performed in the following systems:
- ITLC-SA plates, 0.05 M sodium citrate (pH=5.5). Under these conditions the unbound lutetium or gallium migrate with the solvent front while the other components remains at the origin.
- ITLC-SA plates, MeOH : 1 M ammonium acetate (1:1 v:v). Under these conditions the unbound lutetium or gallium remains at the origin while the other components migrate with the solvent front.

5.3. In vitro stability

The radiolabelled preparation was stored at room temperature in 5 ascorbic acid solution up to 19 hours for ^{177}Lu- DOTA-[Thi^{8},Met(O$_2$)^{11}]-substance P. The stability of preparations (described as the radiochemical purity, RCP) was determined by HPLC and SepPack separation.

6. Results

DOTA-[Thi^{8},Met(O$_2$)^{11}]-substance P was stable in the applied radiolabelling conditions. No product degradation was observed in HPLC (Fig 7 and 8). The radiolabelling yield for ^{177}Lu- DOTA-[Thi^{8},Met(O$_2$)^{11}]-substance P was higher then 98 % with specific activity around 17 GBq/mg of peptide. The ^{177}Lu- DOTA-[Thi^{8},Met(O$_2$)^{11}]-substance P was stable (RCP higher than 95%) at RT up to 19 h (Fig. 9).

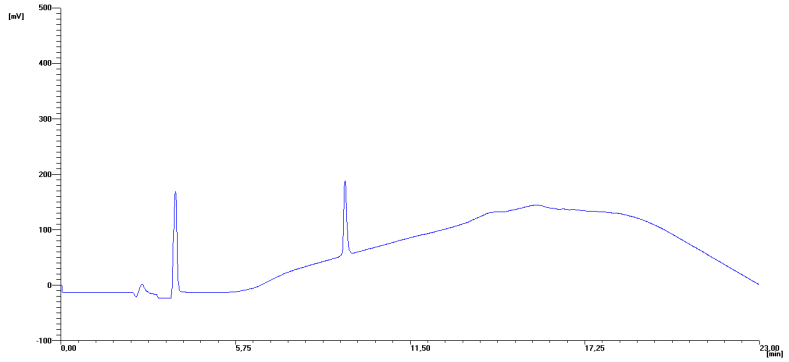

FIG. 7. HPLC-UV profile of DOTA-[Thi8, Met(O2)11]-substance P before labeling.

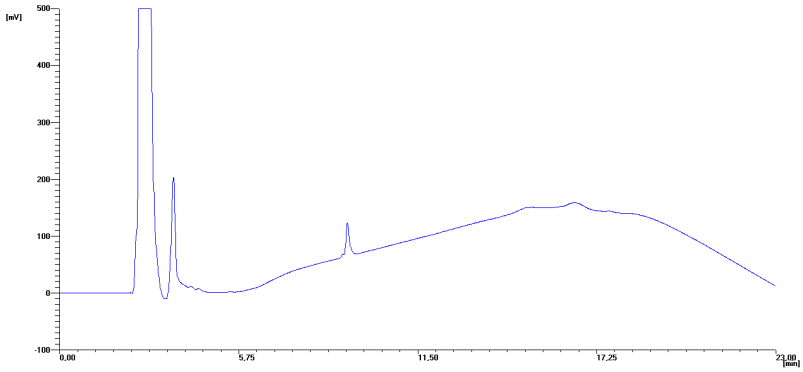

FIG. 8. HPLC-UV profile of DOTA-[Thi8, Met(O2)11]-substance P after labeling

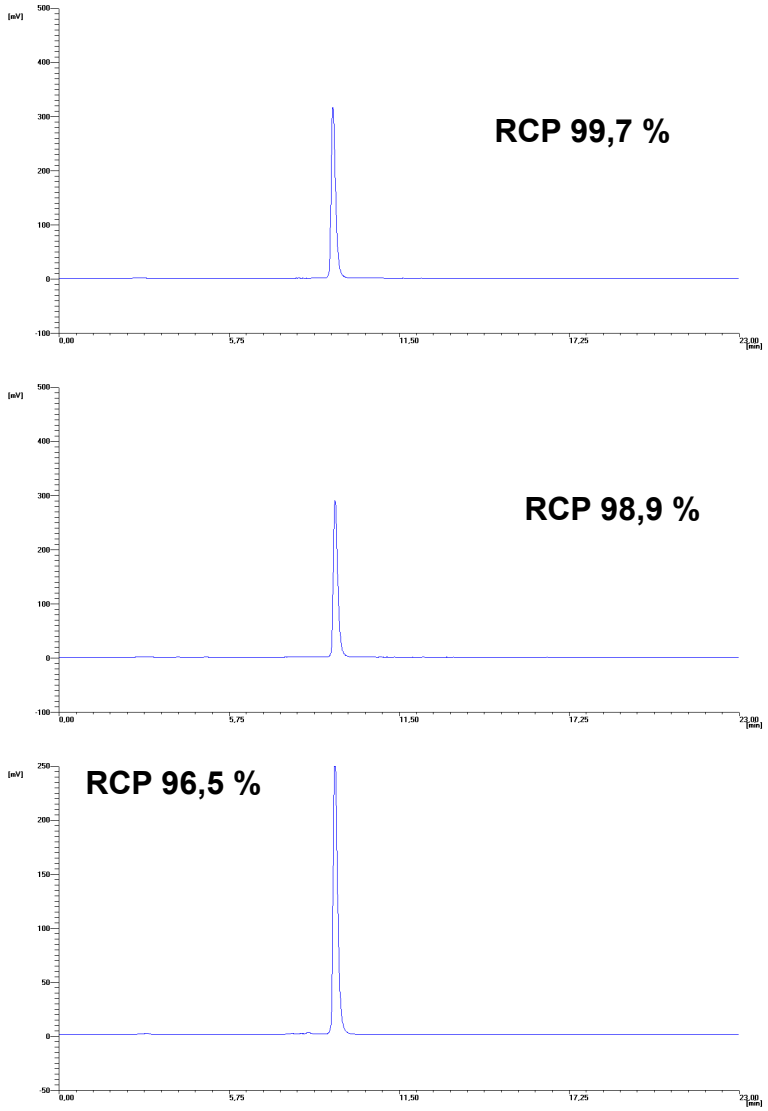

FIG. 9. The Stability of ^{177}Lu-DOTA-[Thi^{8}, Met(O$_2$)^{11}]-substance P at RT 0, 3.5 and 19 hour after labeling.

Radiochemical purity of ^{68}Ga-DOTA-[Thi^{8}, Met(O$_2$)^{11}]--substance P determined by HPLC was in the range from 80.5 to 98.2% (average 90.6%) in 6 consecutive labeling runs in the labeling procedure lasting 20-25 min. Fig.4 presents typical radiochromatogram. In case of TLC methods similar results were obtained. From 81.1 to 98.3% (average 90.9%) for 0.05M sodium citrate and from 83.2 to 99.7% (average 92.2%) for MeOH : 1M ammonium acetate (1:1 v:v). Fig.5 presents the distribution of radioactivity on TLC strips.

High labeling yields and high radiochemical purity of ^{68}Ga-DOTA-[Thi^{8}, Met(O$_2$)^{11}]-substance P allowed its use in patients. Figure 6 presents the PET images acquired directly after administration of therpeutic dose.

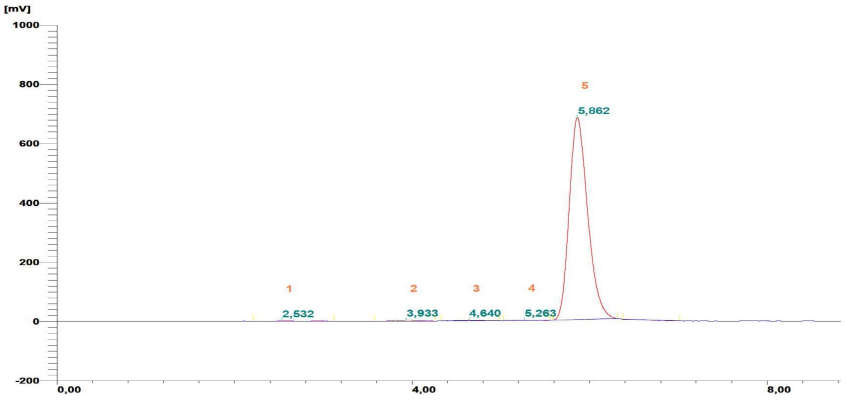

FIG. 10. HPLC chromatogram of ^{68}Ga-DOTA-[Thi^{8}, Met(O$_2$)^{11}]-substance P.

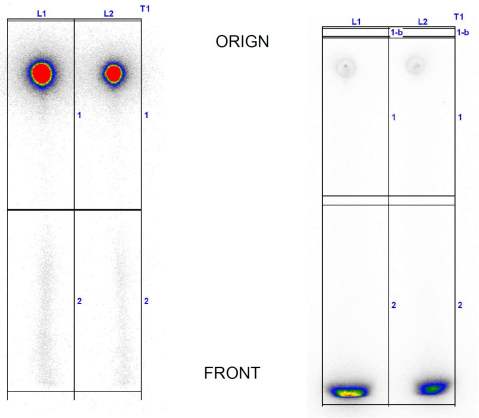

FIG. 11. TLC of ^{68}Ga- DOTA-[Thi^{8}, Met(O$_2$)^{11}]-substance P, (left) ITLCSG and 0.05M sodium citrate, (right) ITLC-SG and MeOH : 1M ammonium acetate (1:1 v:v).

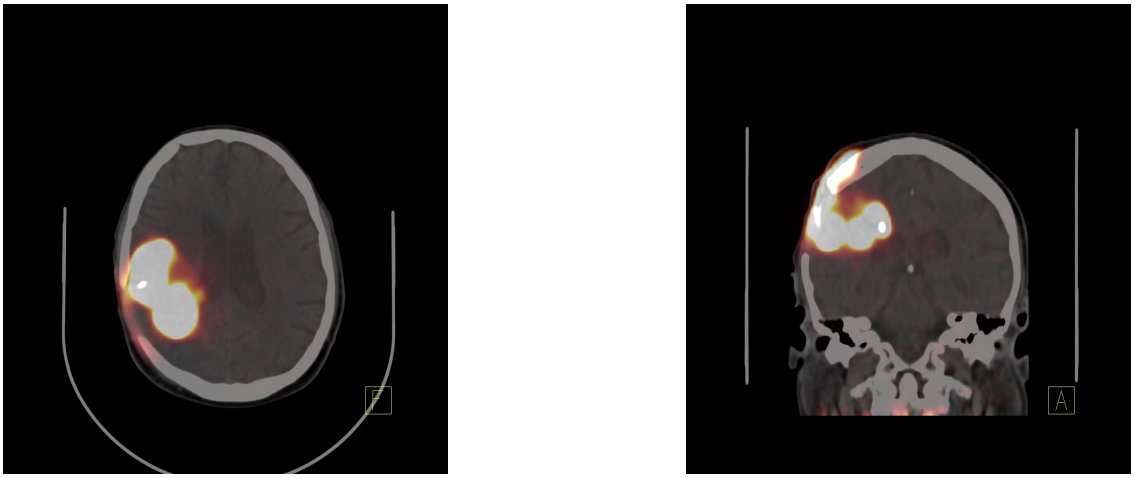

FIG. 12. Post-therpeutic PET images of ^{68}Ga-DOTA-[Thi^{8}, Met(O$_2$)^{11}]-substance P co-injected with ^{213}Bi-DOTA-[Thi^{8}, Met(O$_2$)^{11}]-substance P.

TABLE 3. Summary of radiochemical purity results of ^{68}Ga-DOTA-[Thi^{8}, Met(O$_2$)^{11}]-substance P

Labelling	HPLC	TLC	
		ITLC-SG, MeOH/1M ammonium acetate	ITLC-SG, 0.05M sodium citrate
1	87,5	88,1	91,2
2	80,5	81,1	83,2
3	98,2	98,3	99,7
4	95,7	95,6	97,1
5	92,5	92,9	93,3
6	89,4	89,2	91,3
Mean	90,6	90,9	92,6

Conclusions on DOTA-Substance-P

This study is performed in collaboration with Medical University of Warsaw in the therapy of glioma patients. Study extension to ^{177}Lu-DOTA-[Thi^{8}, Met(O$_2$)^{11}]-substance-P is planned.

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SAUDI ARABIA

Research and Development of Lutetium-177 based radiopharmaceuticals for targeted radionuclide therapy of cancer

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Abstract. The main purpose of this research project was to develop ^{90}Y and ^{177}Lu-labeled bioactive compounds (antibodies and peptides) for targeting of human cancers. A number of peptides have been prepared by solid-phase peptide synthesis according to Fmoc/HBTU chemistry. After radiolabeling with ^{177}Lu radionuclide, these compounds will be evaluated for their potential as radiotherapeutic agents. The results of these evaluations will be discussed.

1. Introduction

Conventional chemotherapy is restricted by intrinsic multidrug resistance of cancerous cells, and the toxic side-effects resulting from high therapeutic doses necessary for efficacy. With the advent of the 21st century, a deeper understanding of cancer turned the focus of cancer research toward a more selective, targeted approach that would eventually produce anticancer therapies with fewer side-effects and improved therapeutic efficacy [1]. One of the various approaches for targeted cancer therapy is based on findings that receptors for certain peptides are overexpressed on a variety of human cancers. These receptors serve as potential molecular targets for cancer diagnosis and therapy with radiolabeled peptides. Peptide-based radiopharmaceuticals, which bind to tumor receptors with high affinity and specificity, hold great promise in nuclear oncology [1-3].

Tumor targeting with radiolabeled peptides is gaining enormous acceptance in nuclear oncology [2, 3]. This is because of the favorable pharmacokinetics and specific tumor targeting characteristics, together with the overexpression of their receptors on tumor cells, makes these receptor binding peptides powerful biological tools for the diagnostic imaging and targeted radiotherapy [2]. Among the most clinically-relevant peptide receptor systems, bombesin (BN) peptide receptors are of great interest, because of the overexpression of their receptors on various important and frequently occurring human cancers including prostate, breast and small cell lung cancer. The high expression in cancer cells and low expression in normal tissues makes BN peptides attractive agents for targeting BN receptor-expressing cancers [2, 3]. These features also provide the possibility to use BN-like peptides for therapy when labeled with therapeutic radionuclides. Additionally, upon conjugation with cytotoxic drugs, they can be utilized as vehicles for site-specific delivery of cytotoxic drugs into receptor-specific tumor cells [1]. In addition to BN peptides, we will prepare and evaluate HER2 (human epidermal growth factor receptor type 2) also called HER2/neu, a tumor-specific antigen that is amplified and/or overexpressed on several human cancers including breast and ovarian cancer. It was assumed that the synthetic peptides derived from tumor-associated antigens and retaining most or all of the original affinity and specificity to tumor-related antigens can be useful for tumor targeting. The overexpression of HER2/neu in various tumors and relatively low expression on normal tissues makes it an attractive target for tumor targeting with HER2/neu-derived synthetic peptides [4]. Also, s synthetic peptide derived from alpha-fetoprotein, a naturally-occurring human protein produced during pregnancy, which itself has anti-estrogenic and anti-breast cancer activity [5], have been synthesized.

Monoclonal antibodies (MoAbs), designed against various antigens or receptors associated with specific tumor types can be used as targeted carriers of radionuclides to malignant tumors overexpressing either respective antigens or receptors. Since Rituximab will be available soon as a generic product, this coordinated research

project is designed to focus on the development of ^{177}Lu/^{90}Y-labeled DOTA-Rituximab as a therapeutic radiopharmaceutical for the treatment of lymphomas. Formulation of DOTA-Rituximab in a kit form would make it feasible for the routine preparation of ^{177}Lu/^{90}Y- DOTA-Rituximab for radiotherapy.

Radionuclides emitting β-radiations (e.g., ^{90}Y and ^{177}Lu) have shown great therapeutic potential since the emitted particle range exceeds the cell diameter thus more effective killing of tumor cells can be achieved. The radiolanthanides, ^{177}Lu is particularly attractive for use in radiotherapeutic application due to its attractive physical characteristics ($T_{1/2}$ = 6.65 days, $E_{\beta max}$ = 0.497 MeV) and ready availability. With a longer half-life (6.65 days), ^{177}Lu emits not only medium energy β-particles (497 keV), but also a low-abundant γ-photon (208 keV), which enables γ-scintigraphy, tumor dosimetry, disease staging as well as external evaluation of the in vivo targeting efficacy of the administered ^{177}Lu-labeled radiopharmaceuticals [2].

The development of ^{177}Lu-labeled compounds expected to improve antitumor effect by killing peptide receptor-expressing tumor cells more effectively for the better control of the disease. Tumor receptor binding peptides and MoAbs labeled with ^{90}Y or ^{177}Lu have become an important and emerging class of therapeutic radiopharmaceuticals in clinical nuclear oncology. Several ^{90}Y or ^{177}Lu-labeled peptides are currently in clinical trials to determine their therapeutic potential. On the other hand, a number of MoAbs have been already approved by the FDA for immunotherapy of several human cancers.

2. Work plan (work already initiated or in progress)

Within the defined objectives of the current CRP, we see our major contribution towards the design and development of novel peptide-based radiopharmaceuticals for the diagnosis and treatment of human cancers. In addition, we will be focusing our interest in developing DOTA-coupled Rituximab antibody for targeting lymphoma. The main experimental procedures pertaining to this project is outlined below:

1. Preparation of peptide molecules by solid-phase synthesis procedure
2. Coupling of DOTA chelator to the targeting molecules (peptides/Rituximab)
3. Purification and characterization of synthesized molecules
4. Radiolabeling of biomolecules with ^{177}Lu
5. Quality control and HPLC purification procedures of ^{177}Lu-compounds
6. Determination of in vitro stability of radioconjugates in human plasma
7. In vitro binding studies of radiolabeled compounds with tumor cells
8. In vivo animal biodistribution studies in normal mice to determine pharmacokinetics
9. In vivo tumor targeting in nude mice bearing human tumor xenografts

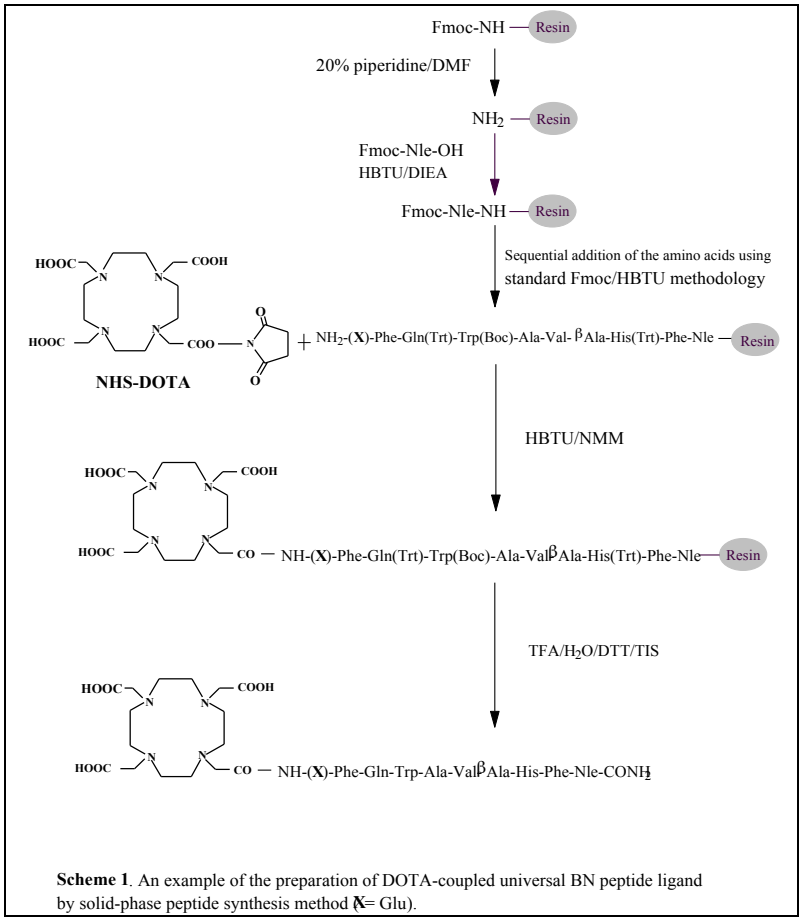
2.1. Synthesis of peptides

All the peptides were prepared by solid-phase peptide synthetic technique on a CS036 peptide synthesizer (CS Bio Co., San Carlos, CA, USA), following standard Fmoc (9-fluorenylmethoxycarbonyl) chemistry on a 0.2 mmol scale. To incorporate the C-terminal amino acids, each Fmoc-protected amino acid (4-fold molar excess compared with the resin) was activated at its α-carboxylic function with an activating reagent, HBTU (*O*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), in the presence of DIEA (diisopropylethylamine), and allowed to react for 60-min with the resin-bound amino acid to form a new peptide-bond. The Fmoc-protecting group was removed using a solution of 20% (v/v) piperidine in DMF (*N,N*-dimethylformamide). The remaining Fmoc-amino acids were assembled on the derivatized-resin by repeated cycles of deprotection (with 20% piperidine in DMF), activation (reaction of 4-fold molar excess of Fmoc-amino acids with HBTU and DIEA to form active ester in situ) and coupling of the activated amino acids with the peptide-resin. After stepwise incorporating all the desired amino acids to the sequence, the last N-terminal Fmoc-protecting group was removed and the peptide-resin washed to prepare for condensation with DOTA (Macrocyclics, Dallas, TX, USA).

For conjugation of peptides to DOTA, NHS-DOTA active ester was prepared first by reacting DOTA ligand with NHS (*N*-hydroxysuccinimide) in the presence of DCC (dicyclohexylcarbodiimide) and then condensed with peptide-resin to produce DOTA-coupled peptides (Scheme 1). The peptide-resin was washed thoroughly and dried. Each peptide was then cleaved from the resin, and the other side-chain protecting groups were removed by treating with a cleavage mixture (~5 mL) of trifluoroacetic acid (TFA)/water/dithiothreitol (95:2.5:2.5) for 2 h at room temperature. The resin was removed by filtration and TFA was evaporated under reduced pressure. The fully deprotected crude peptides were obtained by precipitation with cold diethyl ether. The structures of the synthesized peptides were confirmed by mass spectrometry (Waters Micromass Quattro Premier XE, Manchester, UK).

2.2. Conjugation of Rituximab antibody to DTPA

In our initial experiment, Rituximab antibody was conjugated to *p*-SCN-Bn-DTPA (Macrocyclis) using the molar ratios of BFCA to antibody 15:1. The concentration of the antibody for the conjugation reaction is ~5 mg/mL reconstituted in distilled water. The conjugation was performed at pH 8.5-9.0 for 90 min at 37°C. After conjugation, the purification of bioconjugate was done by PD-10 column. Because of the poor conjugation yield, there is a need to optimize the reaction parameters in order to maximize the conjugation yield.



Scheme 1. An example of the preparation of DOTA-coupled universal BN peptide ligand by solid-phase peptide synthesis method (X= Glu).

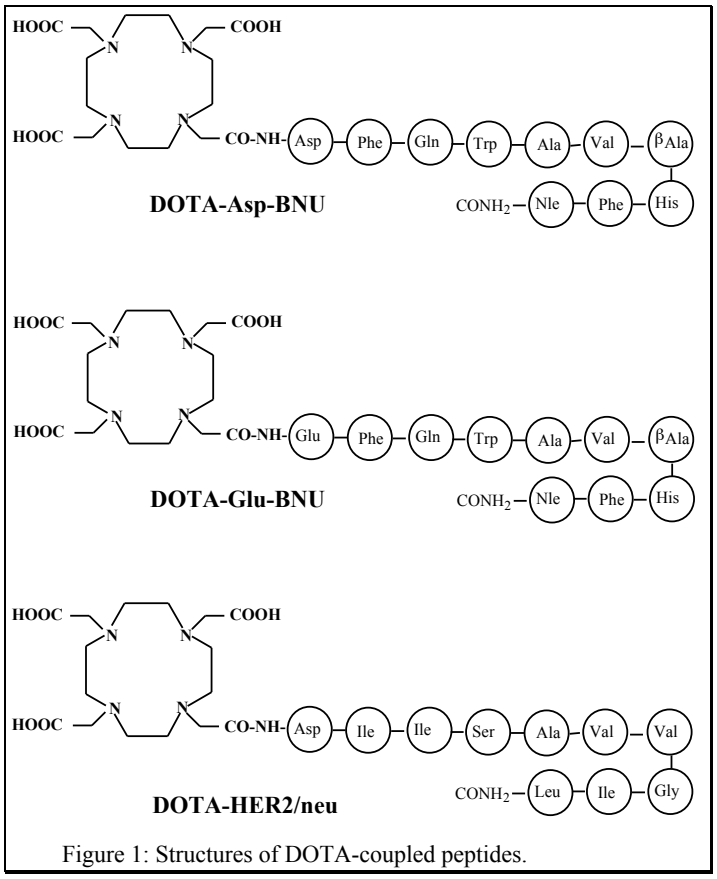


Figure 1: Structures of DOTA-coupled peptides.

2.3. Radiolabeling of biomolecules with Ga-68 and Lu-177

For labeling with Ga-68, ~0.1 mg of each DOTA-conjugate (1 mg/mL in CH_3CN:H_2O) was mixed with 0.4 mL with 0.5 M ammonium acetate buffer followed by 1-2 mCi of Ga-68 (obtained from $^{68}Ge/^{68}Ga$ generator). The pH of the labeling mixture was adjusted to 4.5. The reaction mixture was then heated at 90°C for 35 min. The preparation w filtered through a 0.2-μm pore syringe filter prior to HPLC analysis (Figure 2).

For radiolabeling with Lu-177, 0.5–1.0 mg of DOTA-coupled compounds (1 mg/mL solution in CH_3CN/H_2O) will be labeled with $^{177}LuCl_3$ (~2 μL, 2 mCi), dissolved in 0.1 M HCl. The final pH will be adjusted to 7 with 1.0 M ammonium acetate buffer. The labeling mixture will be incubated at 40°C for 90-min. Labeling conditions, such as incubation time, incubation temperature, and the amount of the ligand to be used will be optimized in order to maximize the labeling yield while reduces the reaction time. Purification and labeling efficiency will then be determined by radio-HPLC analysis.

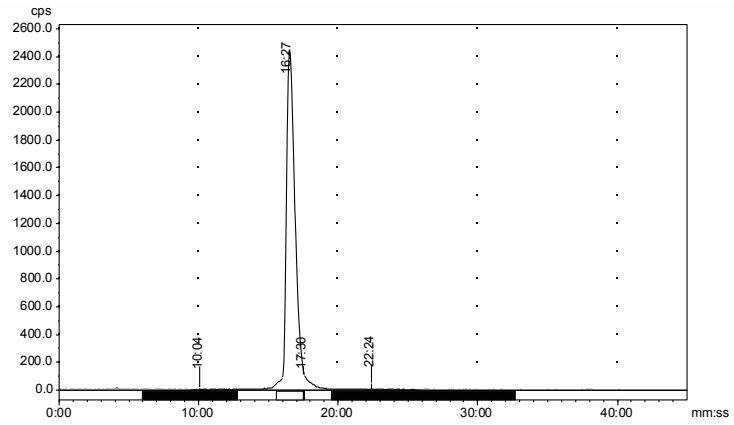


FIG. 2. Radio-HPLC elution profile of ^{68}Ga-DOTA-BN.

2.4. HPLC purification and analysis

HPLC analysis and purification of radiolabeled compounds were accomplished on a Shimadzu HPLC system. Peak separations/isolations were achieved using a C_{18} reversed-phase column (10 μm, 4.6 x 250 mm).

Radiochemical purity was estimated by evaluating radioactivity peaks eluted for each compound from the HPLC column and calculating the area under the peak (ROI). The main peak for each radioconjugate was isolated and dried under a stream of nitrogen. The HPLC-purified compounds were reconstituted in sterile saline for *in vitro* and *in vivo* studies.

2.5. In vitro metabolic stability of radiolabeled peptides

The in vitro metabolic stability was determined by incubating the HPLC-purified radiolabeled compounds with human plasma in duplicate for 1 h at 37°C. Following incubation, the plasma proteins were precipitated with a mixture of CH_3CN/EtOH (1:1) and the sample was centrifuged at a speed of 4000 rpm for 7 min. The su pernatant layer was filtered through a 0.2-μm pore filter and analyzed by HPLC in order to determine the percentage of intact radioconjugate and its potential metabolites (Figure 3).

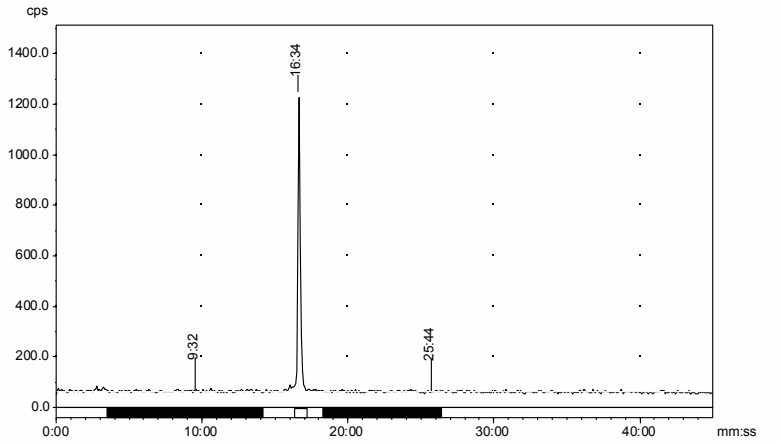


FIG. 3. Radio-HPLC elution profile of ^{68}Ga-DOTA-BN after incubation with human plasma for 60 min at 37°C.

3. Future Work plan

(a) Radiolabeling of biomolecules with Lu-177 will be performed and the reaction conditions will be optimized to achieve high labeling efficiency
(b) Radio-HPLC analysis of radiolabeled compounds will be carried out to obtain carrier-free purified product for in vitro and in vivo studies.
(c) In vitro stability studies will be performed
(d) In vitro cell binding studies using different cancer cell lines
(e) In vivo biodistribution in healthy mice to determine biokinetics
(f) In vivo tumor targeting in nude mice.

4. Research outputs

The expected outputs of this project will be the formulation of protocols for routine preparation and quality control procedures of ^{177}Lu-labeled bioactive molecules, (^{177}Lu-DOTA-BN analogs, ^{177}Lu-DOTA-HER2/neu and ^{177}Lu-DOTA-α-fetoprotein, etc.) for the management and treatment of various cancers in Saudi Arabia as well as settings of the guidelines on quality standardization in the preparation of ^{177}Lu-radiopharmaceuticals for possible human use. In addition, the development and preclinical evaluation of new ^{177}Lu-labeled peptides for the management of cancers, such as breast, ovarian and prostate will be predicted as an outcome of this CRP.

5. Conclusions

The results obtained with these preclinical evaluations will be beneficial in providing useful information for its application in clinical settings, which is the ultimate goal of this project. It is expected that these findings, combined with the availability of the corresponding kit formulation, will favor the subsequent clinical investigation in humans of the new therapeutic radiopharmaceuticals.

ACKNOLEDGEMENT

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SYRIA

Preclinical evaluation and formulation of ^{90}Y-labelled peptides and monoclonal antibody for cancer therapy

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Abstract. The general scope of CRP project is the development of peptides and antibody based radiopharmaceuticals labelled with ^{177}Lu and ^{90}Y to be applied in tumor treatment. We defined many specific objectives such as preparation of ^{177}Lu- or ^{90}Y-labeled DOTA-Rituximab® as therapeutic radiopharmaceutical for the treatment of non-Hodgkin's lymphomas using two different conjugation of the bifunctional chelating agents BFCA such as SCN-Bz-DTPA and SCN-Bz-DOTA for optimization of labelling technique (incubation time, optimal pH, temp., S.A.) of conjugated-MoAb´s and optimize protocols for kit formulation of Rituximab® for radiolabelling with ^{177}Lu and ^{90}Y Rituximab.

Clinical application of peptide receptor radionuclide therapy (PRRT) using Substance P-DOTA for glioblastoma and preparation of ^{177}Lu- or ^{90}Y- labelled DOTA-Substance P for optimization of labelling technique (incubation time, optimal pH, temp, S.A.) of conjugated SP.

1. Radiolabelling of the peptide Substance P with Y-90

Substance P (SP) is an 11-amino acid peptide, which has an important role in modulating pain transmission through neurokinin-1 and -2 receptors (NKr), overexpressed in malignant gliomas. The aim of the present work was to produce a SP analog (DOTA-SP) to be radiolabelled with Y-90, in order to determine the best radiolabeling methodology. The radionuclide Y-90 (half-life, 64 hours) decays exclusively through β-emission suitable for radiotherapy. A high radiochemical purity (> 99%) and high specific activity of DOTA-SP was achieved. Labelling was carried out with 15 µg of SP and 1 mCi (37 MBq) where the mixture was heated at 90 °C for 30 min. The labelling yield was more than 99% and the final labelled product was stable for more than 144 hours in saline and human plasma at temperatures 4° C and -20 °C in saline and HSP, while both at 25°C, the product started to decompose after 72 hrs in both media. The above mentioned condition were used for further labelling procedures.When the reaction time was 30 minutes, the temperature was 90 ^{o}C, the mass of DOTA-SP was 10-15 µg and ^{90}Y activity was 37 MBq (1 mCi) and the radiochemical purity achieved was more than 98%.

2. Material and methods

2.1. Reagents

DOTA-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2 (DOTA-Substance P, PiChem) was provided by IEO-Milan, Italy, and BFCA such as SCN-Bz-DTPA, Rituximab® was provided through IAEA. The precursor $^{90}YCl_3$ was locally obtained from a solvent extraction generator produced in our laboratory. All other chemicals and reagents required for experiments were of analytical grade and purchased from Sigma Aldrich Chemical Co.

2.2. Optimization of radiolabeling conditions

Several studies were carried out to establish the labelling conditions for obtaining the highest yield of labelled DOTA-SP. Conditions were 0.75 - 75 mg od DOTA-SP, 0.2 mol/L sodium acetate buffer (0.3 mL, pH 4.5) and ~100 MBq of $^{90}YCl_3$ [in 0.01N HCl, 37MBq (1 mci), specific activity (0.1mCi/µg] were heated at different temperatures (60 – 95°C) for different times (10 - 35 minutes) and different pH (4-5.5). All reagents of these experiments were prepared with Chelex 100 treated free metal**.**

2.3. Radiochemical purity determination

Thin layer chromatography (ITLC) was applied to determine free yttrium, with citrate/citric acid buffer pH 5.0 as development solvent (R_f of labelled SP-DOTA was 0.1-0.3 and R_f of free yttrium was 0.9-1.0).

3. Stability of radiolabelled SP

3.1 In vivo and in vitro stability

To determine the in vitro stability of ^{90}Y-DOTA-SP the prepared product was stored at 4-20- 25 °C for different times (1 to 9 days) or human plasma samples were spiked with ^{90}Y-DOTA-SP (SA = 0.1 mCi/µg) and incubated for 1, 4 and 24 hours, followed by ITLC analysis. All experiments were performed in triplicate.

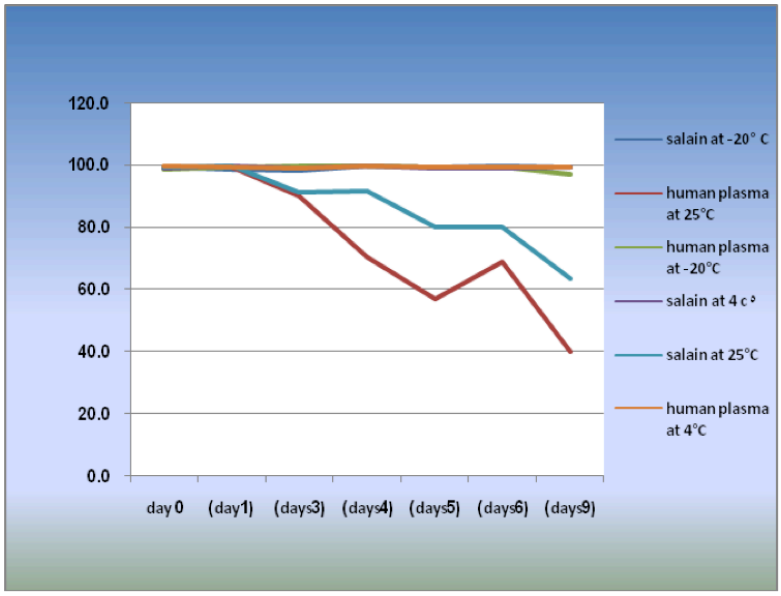


FIG. 1. In vitro stability of radiolabelled SP-DOTA for different times.

	0 day	(1day)	(3days)	(4days)	(5days)	(6days)	(9days)
saline at -20° C	99.6	98.7	98.5	99.9	99.6	100.0	99.6
human plasma at 25°C	99.4	99.5	90.3	70.6	57.1	69.2	40.1

human plasma at -20°C	98.7	99.5	99.9	99.9	99.4	99.4	97.3
saline at 4 c°	99.1	99.9	99.2	100.0	99.2	99.2	99.4
saline at 25°C	99.2	99.7	91.1	91.7	80.2	80.2	63.5
human plasma at 4°C	99.8	99.6	98.9	99.9	99.4	99.4	99.6

3.2. Animals (in vivo)

4 to 6 week old male rat were injected in the lateral tail vein. and blood samples were taken , centrifuged to get plasma samples, for different times (1,3,8,16,20,24 hours), and followed by ITLC analysis. All experiments were performed in triplicate.

3.3. Human plasma (in vitro)

To determine the stability of 90Y-DOTA-SP the preparation was stored (at 37)°C using incubator for different times (1, 3, 8, 16, 20, 24 hours), human plasma samples were spiked with ^{90}Y-DOTA-SP (SA=0.1mci/µg) and followed by ITLC analysis. All experiments were performed in triplicate.

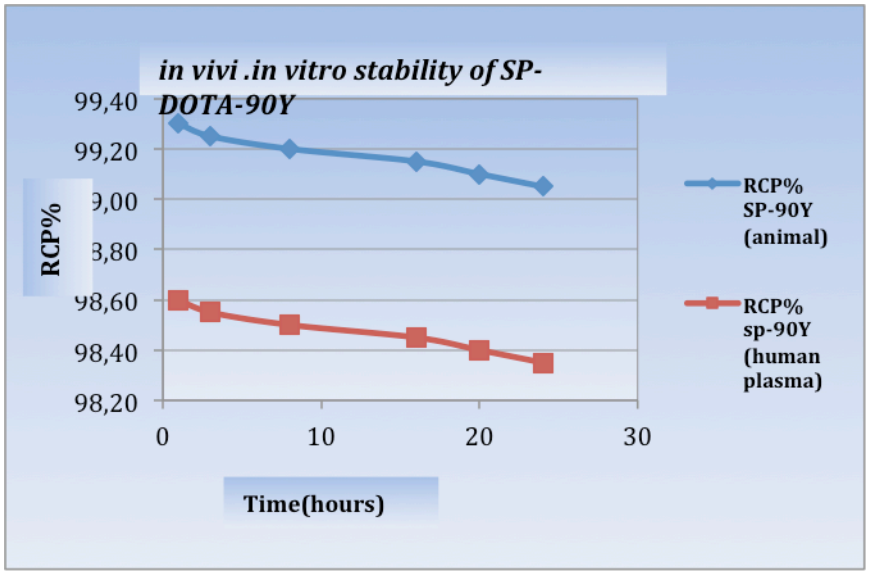
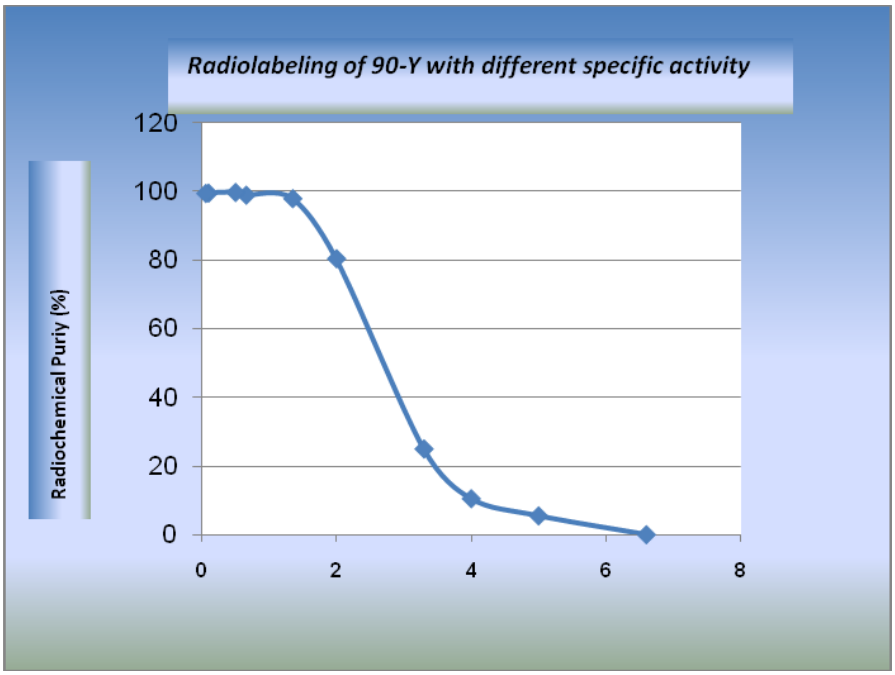


FIG. 2. Radiolabelling purity of Sp-DOTA-90Yin vivo and vitro.

4. Results

Different Substance P masses (0.2-0.75 µg) were radiolabel using 37 MBq (1 mCi) of radionuclide and the results are shown in FIG. 1. High radiolabeling yields (> 95%) were achieved when 0.74, 1.5, 2, 10, and 15 µg of DOTA-SP reacted with 1-mCi Y-90. When the mass of DOTA-SP reduced to 0.3 µg the radiochemical purity decreased to 25 and no radiolabel reaction occurred using 0.2and 0.15 µg of the peptide.



Ratio (mci/µg)	SA (mCi/µg)	RCP%
(mci/0.15µg)	0.15	0
(mci/0.2 µg)	0.2	5.5
(mci/0.25 µg)	0.25	10.5
(mci/0.3 µg)	0.3	25
(mci/0.5 µg)	0.5	80.5
(mci/0.74 µg)	0.74	98
(mci/1.5 µg)	1.5	99
(mci/2 µg)	2	99.8
(mci/10 µg)	10	99.6
(mci/15 µg)	15	99.5

FIG. 3. Radiochemical purity at different conditions using different ^{90}Y/SP ratios (mCi/µg). The reactions were performed at 90°C for 30 minutes. At **1***mCi/15 µg a radiochemical purity of 99.5 was obtained.*

4.1. Study of radiolabelling conditions

Several studies were done to establish the labeling conditions for obtaining the highest yield of labelled DOTA-SP (0.2 - 15 µg), 0.2 mol/L sodium acetate buffer (0.3 mL, pH 4.5) and ~37 MBq of $^{90}YCl_3$ (in 0.01N HCl, 37MBq (1 mCi), specific activity 0.5-15 mci/µg) were heated at different temperatures (60 – 95°C) for

different times (10 - 35 minutes). All reagents of these experiments were prepared with Chelex 100 treated free metal.

4.2. Effect of incubation time

The effect of incubation time on radiochemical purity of ^{90}Y-DOTA-SP was investigated. Radiochemical purity (%) at different times (10, 20, 25, 30 and 35 minutes) was measured and the results are shown in Fig. 3. High radiolabeling yields (> 95%) were achieved when the incubation temperature was between 20 -35 °C .

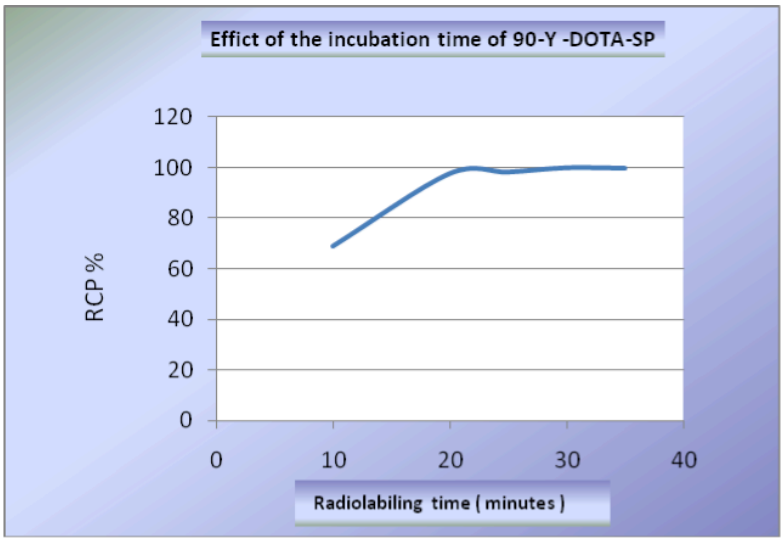


FIG. 4. Effect of the incubation time on radiochemical purity of 90Y-DOTA-SP.

TABLE 1. Effect of the incubation time on radiochemical purity of ^{90}Y-DOTA-SP.

Radiolabiling time (min)	Radiochemical purity %
10	69
20	97.6
25	98.2
30	99.9
35	99.7

4.3. Effect of different temperatures

The labelling conditions for obtaining high yield of labelled substance P, when the temperature between (70-95° C).

TABLE 2. Effect of temperature on radiochemical purity of ^{90}Y-DOTA-SP

Temperature (°C)	RCP %
60	80.4
70	95.9
80	99.5
90	99.9
95	99.5

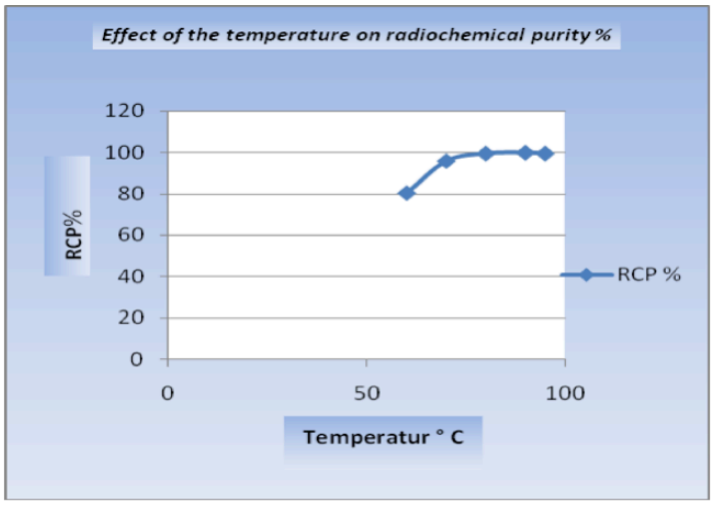


FIG. 5. Effects of the radiolabelling at different temperatures on the radiochemical purity.

4.4. Effect of pH

Labelling conditions showed high yield of labelled substance P, at Ph values between 4,5-5,5

Final (pH)	RCP%
4	79
4.5	99.1
5	98
5.5	98.5

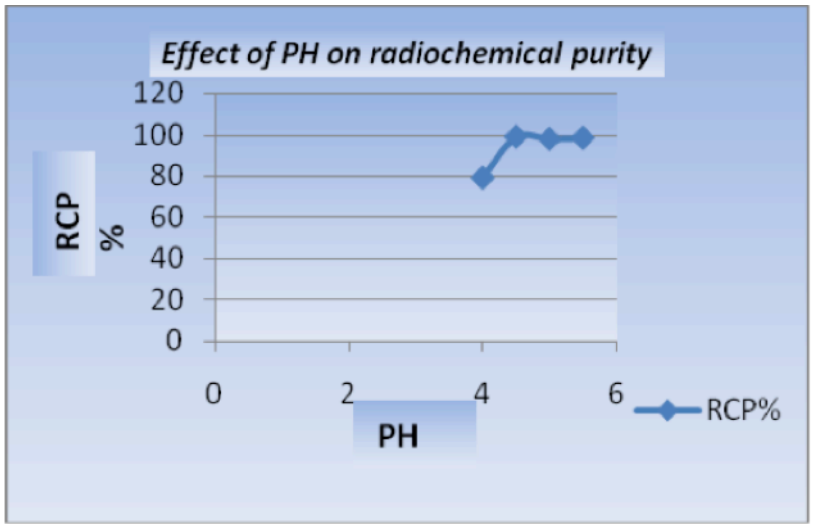


FIG. 6. Effect of the radiolabelling at different pH on radiochemical purity.

5. ^{90}Y-monoclonal antibodies

Anti-CD20 monoclonal antibody (Rituximab®) has become a standard treatment for relapsed or refractory CD20-positive low-gradenon-Hodgkin's lymphoma (NHL), along with Chemotherapy. (Rituximab®) is a chimerical 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.

Over recent years, radio immunotherapy has been used in the treatment of CD20+lymphoma, specifically low-grade NHL that has relapsed or refractory.

Standard therapy is treated. Radio immunotherapy is used alone or in combination with other therapies with the goal of improving efficacy. For this purpose beta-emitting radioisotopes are coupled to anti-CD20 antibodies. Lymphocytes and lymphoma cells are highly radiosensitive.

5.1. Preparation and characterization of ^{90}Y-Rituximab

5.1.1. Material and methods

Chimeric anti-CD20 rituximab monoclonal antibodies were provided by Roche (Roche Pharma Schweiz, Basel,Switzerland). The p-SCN-benzyl-DOTA (Back-DOTA) and The 2-(4-isothiocyanatobenzyl)-6-methyl-diethylenetriamine pent acetic acid (1B4M-DTPA, also known as MX-DTPA) from IAEA, Austria.

The radionuclide ^{90}Y was obtained from ^{90}Sr/^{90}Y generator in our Lab consisting of Sr-Spec Resin(crown ether) packed in three columns for separation and purification of ^{90}Sr/^{90}Y.

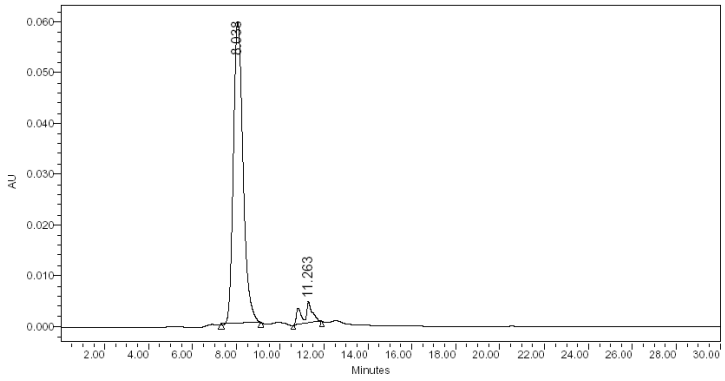
All other reagents were purchased from Aldrich or Sigma, The centrifuge filter devices Amicon Ultra-15filter (Millipore, MWCO 30, 000).

5.1.2. Preparation of Rituximab+MX-DTPA and Rituximab+DOTA-NCS.

1-Buffer sodium carbonate (0.2 M) pH = 9.0.
2-Buffer ammonium acetate (0.25 M), pH = 5.53; buffer ammonium acetate (0.25 M), pH = 7.5.

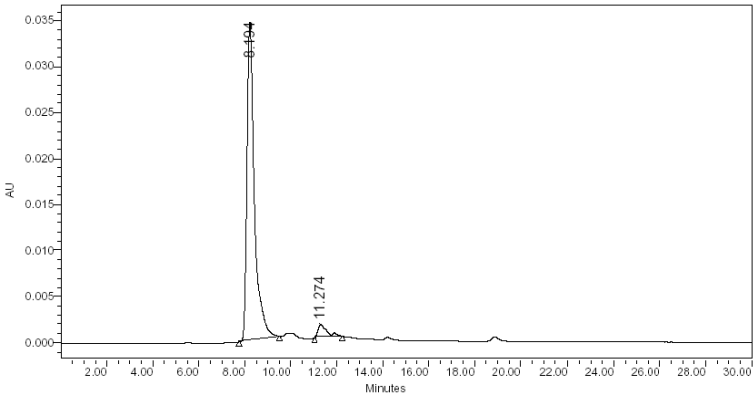
Two different kind of chelating agents were available for coupling reaction: a) MX-DTPA and DOTA-SCN. Both conjugations have been carried out at a molar ratio chelating/antibody of (10:1). Different specific activity (10 , 20 , 25 mci /mg) have been studied.

Commercially available Rituximab monoclonal antibody and DOTA-NCS were used. 428 µl containing (3.0mg) mAb after removing the essential buffer using centrifuge with condition (4000 rpm at 4^{o}C) was divided in two equal aliquots.Then these have been dissolved in carbonate buffer 0.2M at pH = 9.0 using sodium carbonate(0.2M) as buffer solution pH=9. The reaction was allowed to proceed over night at room temperature under vigorous stirring, then the buffer was changed to sodium acetate buffer 0.5M at pH = 7, which is more suitable and the final buffer filtered to obtain 1ml. Inject the sample on the HPLC.



	RT (min)	Area (V*sec)	% Area	Height (V)	% Height
1	8.038	1822992	93.58	59583	93.35
2	11.263	124974	6.42	4246	6.65

FIG. 7. Conjunction of Rituximab with 2(-4isothiocyanatobenzyl)-6-methyl-diethylenetriamine penta acetic acid (1B4M-DTPA, also known as MX-DTPA) on HPLC.



	RT (min)	Area (V*sec)	% Area	Height (V)	% Height
1	8.194	778609	95.32	34656	96.04
2	11.274	38222	4.68	1428	3.96

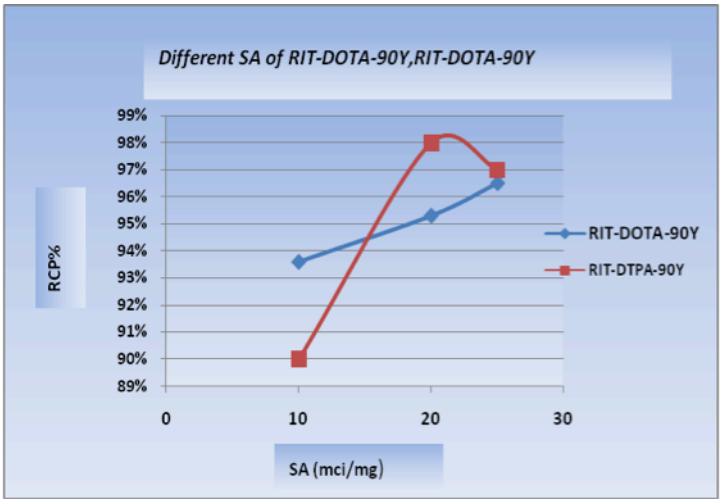
FIG. 8. Conjugation of Rituximab with p-isothiocyanato benzeyl DOTA(p-NCS-Bz-DOTA) Rituximab+DOTA-NCS

The ^{90}Y was obtained from $^{90}Sr/^{90}Y$ generator in our lab consisting of Sr-Spec Resin(crown ether) packed in three columns for separation and purification of $^{90}Sr/^{90}Y$. The generator was loaded with about 50 mci of ^{90}Sr and the yield of ^{90}Y was about 45 mci which means more than 90% elution efficiency.
Each labeling was carried to reach different specific activity (SA) as detailed below.

(1) SA: 10 mCi/1mg
88 µl of Y^{90} activity 3mCi, pH = 5.
100 µl of Rituximab-DOTA in acetate (0.3mg/100µl), pH = 7.5.
100 µl of (0.01 µl DTPA, pH = 7.5).
Incubate at 42°C (for 1hr).

(2) SA: 20 mCi/1mg.
105 µl of Y^{90} activity 6 mCi, pH = 5.
100 µl of Rituximab-DOTA in acetate (0.3 mg/100µl), pH = 7.5.
100 µl of (0.01 µl DTPA, pH=7.5).
Incubate at 42°C (for 1 hr).

(3) SA: 2 5mCi/1mg.
140 µl of Y^{90} activity 7.5mCi, pH = 5 .
100 µl of Rituximab-DOTA in acetate (0.3mg/100µl), pH = 7.5
100 µl of (0.01 µl DTPA pH = 7.5).
Incubate at 42 °C (for 1hr).



SA (mCi/1mg)	RCP%	RCP%
	RIT-DOTA-90Y	RIT-DTPA-90Y
10	93.60%	90%
20	95.30%	98%

25	96.50%	97%

FIG 9. Different SA of RIT-DOTA-^{90}Y, RIT-DTPA $^{-90}$Y

5.3. Quality control

5.3.1. High Pressure liquid Chromatography

Quality control of radio pharmaceutical is carried out by high performance liquid chromatography column: (2.1*200 mm) Hypersil AA-ODS – 5 micron, Flow: 0.4 m/min, λ = 254nm Mobile phase : 95% NaCl, 5% ACN)-20 min. The analysis (HPLC) showed an overall radiochemical purity of RCP ≥ 95-98%.

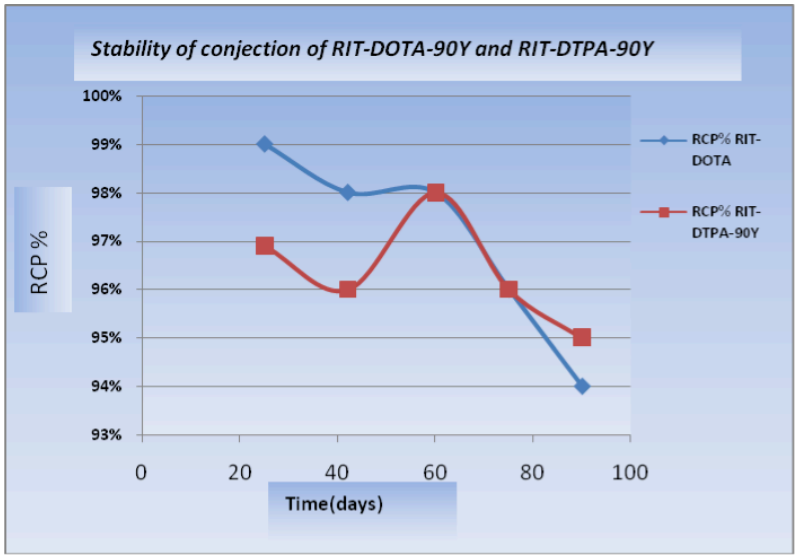
5.3.2. Thin Layer Chromatography

ITLC-SG (instant Thin Layer Chromatography-Gelman Sciences) paper using with several mobile phases (Saline 0.1 M pH=7 , Methanol: ammonia 3:2 , Methanol: ammonium acetate 1:1). ITLC radioactivity was analyzed by cutting the strips in two segments and the radioactivity was measured by LS counter (Beckmam, USA) the results were calculated and the radio chemical purity was Showed that both reactions were at (SA 10, 20,25 mci /mg) successful thus obtaining labeling yield of 99.885% and 99.5%.

5.4. Stability studies

5.4.1. Stability of conjunction of RIT-DOTA- and RIT-DTPA

The stability of conjugates of monocular antibody with two different chelating agents and after storage at 1, 25, 42, 60, 75, 90 days was evaluated by ITLC after injecting each sample on HPLC. Table below showed the radio labeling with ^{90}Y for 3 months storage of these conjugates.

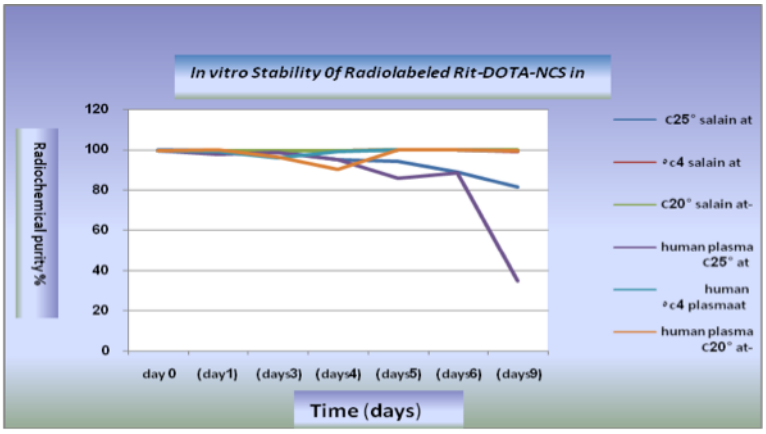


Time(days)	RCP%	RCP%
1	RIT-DOTA	RIT-DTPA
25	99%	97%
42	98%	96%
60	98%	98%
75	96%	96%
90	94%	95%

FIG. 10. Stability of conjugates of RIT-DOTA- and RIT-DTPA during 90 days.

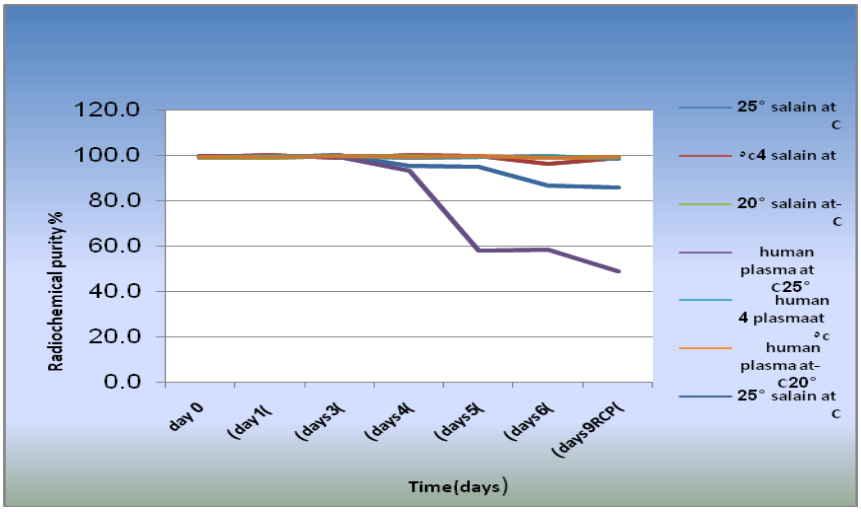
5.4.2 In vitro

The stability of labeled monoclonal antibody was evaluated by ITLC after storage at +4ºC,-20ºC and incubate at room temperature in saline and human plasma for 9 days for both Rituximab+ MX-DTPA, Rituximab+DOTA-NCS.



	(0 day)	(1 day)	(3 days)	(4 days)	(5 days)	(6 days)	(9 days)
saline at 25° C	99.9	99.3	99.0	94.9	94.3	88.9	81.2
saline at 4 c°	99.6	99.3	99.5	99.6	99.9	99.8	98.9
saline at-20° C	99.5	99.4	99.5	99.4	99.9	99.8	99.7
human plasma at 25° C	99.4	97.5	98.5	95.0	85.6	88.2	34.9
human plasma at 4 c°	99.4	99.1	95.8	99.2	99.8	99.9	99.3
human plasma at-20° C	99.3	99.8	96.4	90.4	99.7	99.9	99.5

FIG. 11. Stability of radiolabelled Rit-DOTA-NCS.



0 day	(1day)	(3days)	(4days)	(5days)	(6days)	(9days)
99.9	99.3	99.0	94.9	94.3	88.9	81.2
99.6	99.3	99.5	99.6	99.9	99.8	98.9
99.5	99.4	99.5	99.4	99.9	99.8	99.7
99.4	97.5	98.5	95.0	85.6	88.2	34.9
99.4	99.1	95.8	99.2	99.8	99.9	99.3
99.3	99.8	96.4	90.4	99.7	99.9	99.5

FIG. 12. Stability of radiolabelled Rit-DOTA-NCS.

5.4.3. Animals (in vivo)

4 to 6 week old male rat were injected in the lateral tail vein. and blood samples were taken , centrifuge to get plasma samples. The stability of labeled monoclonal antibody for both was evaluated by ITLC different times. The radiochemical purity was not less than 98 % during 24 day.

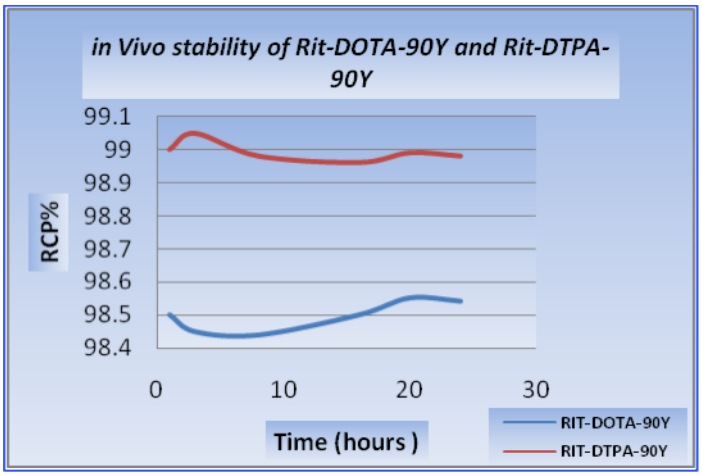


FIG. 13. Stability of RIT-DOTA-^{90}Y and RIT-DTPA-^{90}Y.

APPENDIX B

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