Physioacta, 2014; 8(2): 113-120

Rituximab-immunoconjugate kit-formulations for NHL radioimmunotherapy

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Abstract
Radioimmunotherapy (RIT) of Non-Hodgkin's lymphoma (NHL) is said to be more advantageous compared to unlabeled therapeutic antibodies. To this date, radiolabelled murine anti-CD20 mAbs, Zevalin® and Bexxar® have been approved for imaging and therapy. A preparation containing rituximab, chimeric mAb immunoconjugate suitable for Lu-177 labeling, could provide better imaging and therapeutic profile at the same time. This study was conducted to evaluate prepared lyophilized formulations of three rituximab immunoconjugates, intended for immediate Lu-177 labeling, for imaging and therapy.
The results showed preserved antibody structure and suitability for successful radiolabeling with over 95% radiochemical purity, encouraging further evaluation experiments.
Key words: Rituximab, kit-formulation, radiolabeling, Lu-177, Non-Hodgkin's lymphoma.

Кит-формулации на имуноконјугати на ритуксимаб за радиоимунотерапија на не-Хочкин лимфом

Апстракт
Радиоимунотерапијата (РИТ) на не-Хочкин лимфомите (НХЛ) се смета за понапредна во споредба со необележаните терапевтски антитела. До денес, радиообележаните глувчешки анти-CD20 моноклонални антитела, Zevalin® and Bexxar® се одобрени за дијагностички и терапевтски цели. Препарат кој содржи ритуксимаб, односно конјугат на ова химерно моноклонално антитело, погодно за обележување со Lu-177, би можело да обезбеди подобар профил на истовремена дијагностика и терапија. Ова истражување беше спроведено за да се оценат подготвени кит- формулации на три имуноконјугати на ритуксимаб, наменети за непосредно обележување со Lu-177 за дијагностика и терапија. Добиените резултати покажаа зачувана структура на антителото и погодност за радиообележување со висок процент на радиохемиска чистота, над 95%, охрабрувачки за понатамошни истражувања.
Клучни зборови: ритуксимаб, кит-формулација, радиообележување, Lu-177, не-Хочкин лимфом.

Introduction

Although when in the earliest 2000s, Bexar® and Zevalin® have been approved for RIT of NHL by the Food and Drug Agency (FDA), it was done with very encouraging results and a high percentage of patients who entered the long-term remission [1], still today, there are no new radioimmunoconjugates approved for RIT of solid tumors. The above mentioned fact is due to physical, chemical, biological, clinical, regulatory and financial constraints that limited the progress of these drugs [2, 3]. Accordingly, a development of new formulations of immunoconjugates labeled with radioisotopes is essential for progress in therapy/diagnostic of NHL. Non-Hodgkin's lymphoma is a form of blood cancer with origin in lymphatic system. More than 90% of B-cell lymphoma cells express CD20 receptor which has proven to be an excellent target for the treatment and investigations for treatment of NHL have been based on the development of antibody against these antigens [4, 5].
Results of these investigations have led to developing of drugs such as rituximab, other anti-CD20 monoclonal antibodies (mAbs) labeled with ^{111}In (^{111}In-ibritumomab) for imaging, ^{90}Y (^{90}Y-

ibritumomab, Zevalin) for therapy and ^{131}I (^{131}I-tositumomab, Bexxar) for imaging and therapy, which have been approved for use in patients with NHL [6-10]. Rituximab radioimmunoconjugates are among mostly investigate potential therapeutics for RIT. Various radionuclides, among them ^{90}Y, ^{111}In, ^{64}Cu, ^{153}Sm, and ^{177}Lu [10-16], attached to antibodies have been described or are under development. A number of chelating agents have been investigated for labeling antibodies with radioisotopes, with various derivatives of the acyclic agent diethylene triamine pentaacetic acid (DTPA) and the macrocyclic agent 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) being the most widely investigated [17].

Therapeutic monoclonal antibodies are very complex molecules. Therefore, their integrity is of essential importance for the physico-chemical stability in the preparations and subsequent immunological and therapeutical potential. Monoclonal antibodies as protein molecules intended for therapy are often formulated in aqueous solution to allow ease of use, but because aqueous environment can accelerate many degradation processes [18, 19], the common approach of stabilization of these therapeutic preparations is lyophilization. The lyophilization process is a process which can assure product's sterility and stability requirements [20].

In order to obtain ^{177}Lu-rituximab radioimmunoconjugates for use in diagnostic/therapeutic purposes, different bifunctional chelating agents-anti-CD20 (rituximab) (BFCA-rituximab) were labeled with non-radioactive Lu for preliminar chemical characterization.

While preparations of different derivatives of DOTA and DTPA radioimmunoconjugates has been reported [17], chemical characteristics, stability and biodistribution of the prepared radioimmunoconjugates have not been explained in details.

The main goal was to determine the suitability of preparation and labeling procedures for obtaining kit-formulation ready for radioactive labeling.

Materials and methods

Materials

p-SCN-Bn-DOTA [2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-tetraacetic acid], p-SCN-Bn-DTPA [2-(4-isothiocyanatobenzyl)-diethylenetriaminepentaacetic acid] and 2-(4-isothiocyanatobenzyl)-6-methyl-diethylene-triaminepentaacetic acid (1B4M-DTPA) with 94 % purity were obtained from Macrocyclics Inc. (NJ, USA). Rituximab was purified from a commercial pharmaceutical sample (Mabthera®), purchased from Roche Co, CA, USA, using ultrafiltration (Ultracel® - 30K, Millipore, Ireland) for concentration and buffer exchange to sterile 0.1 M PBS, pH 8.0.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the mini-gel system (GE Healthcare/Amersham Biosciences).

Conjugation of Rituximab to p-SCN-Bn-DOTA, p-SCN-Bn-DTPA and 1B4M-DTPA

The chelating agents were dissolved in 0.1 M PBS (pH 8.0) to final concentration of 10 mg/mL. Amounts required to give a 20-fold molar excess over the amount of rituximab (10 mg/mL) were added to antibody in 0.1 M PBS (pH 8.0) solution. The mixture was incubated for 16h, at 4°C. Purification of the conjugates was made with ultrafiltration (Ultracel® - 30K, Millipore, Ireland), by washing with 0.05 M ammonium acetate, pH 7.0, until the absorbance in the ultrafiltrate at 280 nm was nearly zero.

Lyophilization Process

The lyophilization was performed using Labconco Free Zone Stoppering Tray Dryer, (USA) using protocol described by Park et al., in 2013 [21], modified to our experience. Briefly, the liquid immunoconjugates were filled in 10 mL type I glass tubing vials using a fill volume of 1 mL and loaded in the freeze-dryer. The temperature was decreased to -40°C and held for 3 h, increased to -15°C, to allow complete crystallization, thus completing the freezing step in 10 h. The primary drying

was performed at temperature of -10°C and the secondary drying at shelf temperature 25°C. Upon finishing the process, the vials were kept at 4°C until analysis.

Labelling the BFCA-rituximab conjugates with non-radioactive Lu

BFCA-rituximab conjugates in a form of freeze-dried preparations were dissolved with 0.9% NaCl, and labeled subsequently with 1.0709 µg $LuCl_3$ [equivalent to maximum tolerated dose (MTD) for ^{177}Lu (4377.1 MBq)] in a total volume of 1mL at pH 7.0, and incubation for 30 min at room temperature (DTPA-rituximab and 1B4M-DTPA-rituximab) and 60 min at 40°C for DOTA-rituximab.

Protein Integrity Test Using SDS-PAGE

SDS-PAGE was performed according to Laemmli protocol [22]. About 5 µL of sample was mixed with 10 µL of sample buffer and boiled for 5 min at 95° C. Approximately 5 µL of each preparation was applied per lane in 12% bisacrylamide under reducing conditions. Coomassie staining (Coomassie Brilliant Blue R-250, Sigma) was used for visualization purposes. As molecular marker Low molecular weight marker (Amersham GE Healthcare) was used.

Radiolabeling of the BFCA-rituximab conjugates with Lutetium-177

The radiolabeling of the freeze-dried immunoconjugate was performed after reconstitution in 0.9% NaCl, in the presence of acetate ions at pH 7.0 with Lutetium-177 with specific activity of 555 GBq/mg, at room temperature. The radiolabeled immunoconjugates were obtained with high radiochemical yield. The radiochemical purity was determined using size-exclusion (SE-HPLC).

SE-HPLC

The obtained radioimmunoconjugates were characterized with SE-HPLC. Apparatus conditions were: column Zorbax Bio Series GF-250; sample volume: 20 µl; mobile phase: 0.9% NaCl flow rate: 1 mL/min; UV detection at 280 nm and radiometric detection.

Results

After lyophilization, the obtained formulations were dissolved in 0.9% NaCl giving clear to very slight opalescent solutions (Figure 1).

a) b)

Figure 1: a) Immunoconjugates (three types) after lyophilization; b) Immunoconjugates (three types) after dissolution.

In order to demonstrate the integrity of the protein and purity after conjugation and lyophilization, SDS-PAGE was performed using 12% bisacrylamide gel. The loaded samples were Rituximab (1 mg/mL, commercial sample), conjugated, p-SCN-Bn-DTPA, p-SCN-Bn-DOTA and 1B4M-DTPA in

reconstituted lyophilized formulation and non-radioactive labeled immunoconjugates. On Fig. 2 and 3 SDS-PAGE patterns for non-labeled and non-radioactive-Lu-labeled conjugates, compared to unconjugated rituximab as control sample are shown.

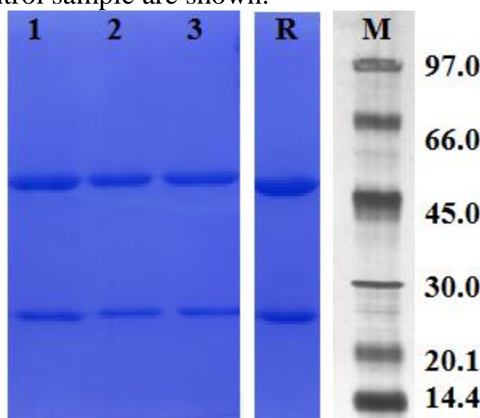

Figure 2: Reducing SDS-PAGE lane patterns for 1) DTPA-rituximab conjugate, after lyophilization, 2) DOTA-rituximab conjugate, after lyophilization, 3) 1B4M-DTPA-rituximab conjugate, after lyophilization, R) rituximab 1 mg/mL, M) molecular marker.

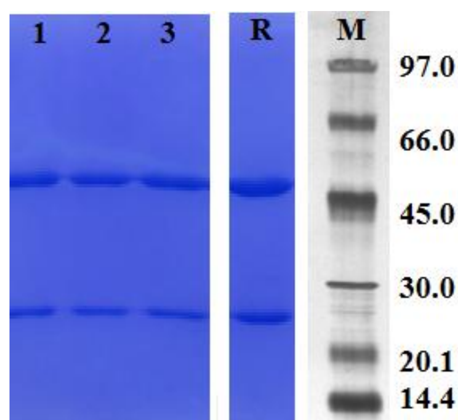

Figure 3. Reducing SDS-PAGE lane patterns for 1) Lu-DTPA-rituximab conjugate, 2) Lu-DOTA-rituximab conjugate, 3) Lu-1B4M-DTPA-rituximab conjugate, R) rituximab 1 mg/mL, M) molecular marker.

The results showed that all BFCA-rituximab conjugates (before and after labeling) were resolved in two distinct Mw species which migrated in two bands (upper band at ~50 kDa and lower band at ~25 kDa). As it is shown in Fig. 2, the reducing SDS-PAGE patterns for rituximab, and BFCA-rituximab immunoconjugates after lyophilization and reconstitution were with very similar intensity. Similar to this, Fig. 3 shows the lane patterns of non-radioactive-Lu-labeled BFCA-rituximab immunoconjugates, showing high resemblance patterns before and after labeling.

The radiolabeling was performed to immunoconjugates with one acyclic, DTPA-rituximab and cyclic, DOTA-rituximab BFCAs after reconstitution of the lyophilized preparation with 0.9% saline. Representative SE-HPLC profiles of the radiolabeled immunoconjugates are shown in Figure 4-5. In the chromatogram obtained, a presence of only one peak is registered, that corresponds to the radiolabeled antibody. The radiochemical purity of the immunoconjugate is over 95%, which meets quality criteria.

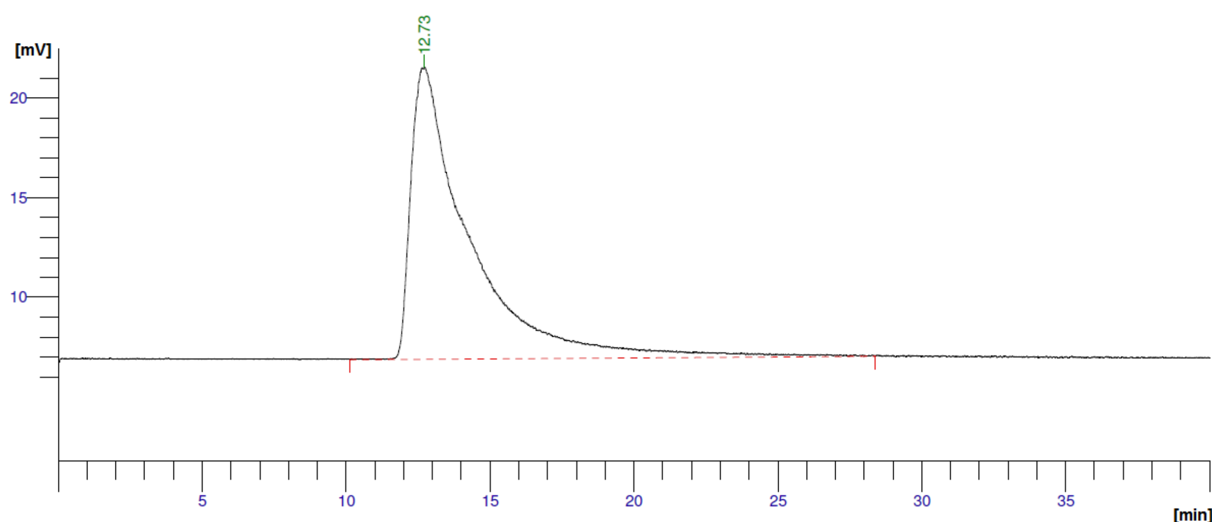

Figure 4: SE-HPLC of ^{177}Lu-DOTA–rituximab

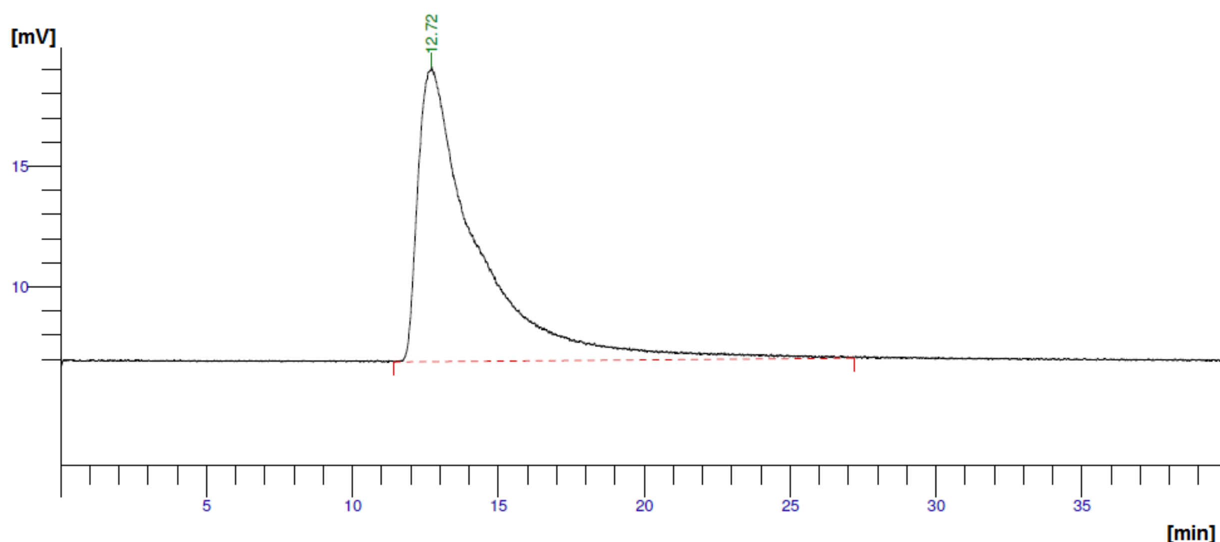

Figure 5: SE-HPLC of ^{177}Lu-DTPA-rituximab

Disscusion

Our study was aimed at assessing physicochemical properties of new lyophilized immunoconjugates intended for immediate labeling with Lu-177, with a possibility to be used in NHL treatment. Indeed, because of its protein nature, rituximab may go through a variety of chemical and physical degradation processes [23]. The preparation of protein therapeutics as lyophilized (freeze-dried) products is often essential to obtain stabile pharmaceutical formulation. The lyophilization protocol used did not affect structure properties and caused no post-lyophilization modification, as shown in the reducing SDS-PAGE lane patterns (Fig. 2), in comparison to the result of commercially available rituximab sample. No clear indication for antibody degradation after labeling with non-radioactive Lu was also registered using reducing SDS-PAGE (Fig. 3). The performed SDS-PAGE electrophoresis in reducing conditions confirm the migration behavior typical for IgG antibodies which are comprised of two identical subunits, each composed of two polypeptide chains: two heavy and two light chains, linked via disulfide bonds [24, 25] The HPLC profiling of all three Lu-177 radioimmunoconjugates, revealed only one peak, which corresponds with the radiolabeled conjugate(s), thus manifesting high radiochemical purity that is essential for the desired biodistribution of the radiopharmaceuticals.

Conclusion

Our results demonstrate that after lyophilization, all three rituximab immunoconjugates remain stable and can bind with Lu-177 radioisotope, thus obtaining products with high radiochemical purity. Regarding these encouraging results, further experiments will be performed order to demonstrate their biological and pharmacological properties. Our results also support the possibility of preparing standardized batches of "ready-to-label" rituximab immunoconjugates in order to develop a new promising radiopharmaceutical for therapy of NHL.

Acknowledgments
This work was supported by IAEA's Coordinated Research Project (CRP): "Therapeutic radiopharmaceuticals based on ^{177}Lu and ^{90}Y labeled monoclonal antibodies and peptides: Development and preclinical evaluations".

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