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RADIOLABELING OF ANTI-CD20 WITH Re-188 FOR TREATMENT OF NON-HODGKIN'S LYMPHOMA: RADIOCHEMICAL CONTROL

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ABSTRACT

The development of tumor-selective radiopharmaceuticals is clinically desirable as a means of detecting or confirming the presence and location of primary and metastatic lesions and monitoring tumor response to (chemo)therapy. In addition, the application of targeted radiotherapeutics provides a unique and effective modality for direct tumor treatment. In this manner the radioimmunotherapy (RIT) uses the targeting features of monoclonal antibody to deliver radiation from an attached radionuclide. Antibody therapy directed against the CD20 antigen on the surface of B-cells is considered one of the first successful target-specific therapies in oncology. The radionuclide rhenium-188 (^{188}Re) is currently produced from the father nuclide tungsten-188 (^{188}W) through a transportable generator system. Because of its easy availability and suitable nuclear properties ($E_{\beta MAX}$ = 2.1 MeV, $t_{1/2}$ = 16.9 h, E_{γ} = 155 keV), this radionuclide is considered an attractive candidate for application as therapeutic agent and could be conveniently utilized for imaging and dosimetric purposes. The purpose of this work is to show the radiochemical control of the optimized formulation (solution) and lyophilized formulation (kit) of labeled rituximab (anti-CD20) with ^{188}Re. Rituximab was reduced by incubation with 2-mercaptoethanol at room temperature. The number of resulting free sulfhydryl groups was assayed with Ellman's reagent. Radiochemical purity of ^{188}Re-rituximab was evaluated using instant thin layer chromatography-silica gel (ITLC-SG). Quality control methods for evaluation of radiochemical purity showed good labeling yield of the antibody.

1. INTRODUCTION

B-cell non-Hodgkin's lymphomas (B-NHL) are a heterogeneous group of lymphoproliferative malignancies with different biological behavior and treatment responses. Most patients with B-cell lymphoma respond well to standard treatment with cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) but patients with relapsed, progressive or transformed low-grade lymphoma still have a poor prognosis [1]. Most B-cell non-Hodgkin's lymphomas express the CD20 antigen and certain characteristics make this antigen an appealing target for monoclonal antibody therapy. CD20 is present on the surface of mature B cells but is not present on B cells precursors, mature plasma cells, or other nonlymphoid normal tissues. It is not shed from the cell surface, and does not internalize upon antibody binding. CD20 antigen is not found free in the circulation; thus an agent that reacts with CD20 is not neutralized before binding with the target cell [2].

Rituximab (RTX) is a human/murine chimeric monoclonal antibody (IgG1 type) engineered by grafting the variable regions targeting the CD20 antigen from a murine anti-CD20 antibody (2B8) on to human constant regions [2]. It specifically binds to the CD20 antigen (with high affinity, approximately $5x10^{-9}$ mol/L) [3] and induces apoptosis, cell mediated

cytotoxicity, complement-dependent cell lyses and cytokine release. Rituximab was developed as a treatment for malignancy and as a single agent has been associated with response rates of 50% to 70% in follicular lymphomas and 15% to 50% in small B-cell lymphoma. When it is added to standard chemotherapy, higher response rates and longer overall survival without clinically significant toxicity are achieved [4]. Based on several studies, immunotherapy became an approved treatment modality for B-cell NHL in 1997, with the United States Food and Drug Administration (US FDA) approval of rituximab for the treatment of low-grade or follicular, relapsed or refractory, B-cell NHL. Less than one year later, rituximab was approved for use in the European Union under the indication of stage III/IV, follicular, chemoresistant, or relapsed (two or more relapses) NHL [5].

Even though rituximab can improve the results of salvage chemotherapy in patients with recurrent disease, there had still a need for additional therapeutic options, since cure is extremely rare. Radiolabeled monoclonal antibodies are examples of these additional options. Indolent lymphomas are known to be radiosensitive, but the utilization of external beam radiation therapy as a treatment modality against lymphoma is limited by toxic effects on normal tissues. So, radioimmunotherapy (RIT) using radiolabeled antibodies directed against specific surface antigens is a new treatment option for lymphomas [1]. RIT is advantageous compared with the use of unlabeled antibody alone, because of the additive effect of radiation-induced cytotoxicity and the ability of the associated radioactivity to kill tumor cells some distance from the bound radiolabeled antibody [6]. With this purpose, in 2002, Yttrium-90 Ibritumomab Tiuxetan (Zevalin; Biogen Idec Inc, Cambridge, MA) became the first radioimmunotherapeutic agent approved by the US FDA for the treatment of patients with relapsed or refractory low-grade, follicular, or transformed B-cell NHL, including patients with rituximab-refractory follicular NHL and in 2003, another radioimmunoconjugate called Iodine-131 Tositumomab (Bexxar; Corixa Corp, Seattle, WA), was approved by the same organ. The anti-CD20 antibodies used in Zevalin (IgG 1 type) and Bexxar (IgG 2a) (ibritumomab and tositumomab, respectively) are both murine antibodies [7-9].

Several characteristics that need to be considered for the selection of a radionuclide for use in RIT are the type of particle and particle energy emitted by the isotope (alpha, beta, gamma, and auger), the physical half-life of the isotope, and the distance traveled by the particle where 90–95% of its energy will be transferred to the cell (linear energy transfer, LET) [5]. Beta particles are tumoricidal over a distance of many adjacent cell diameters and achieve killing of antigen-negative tumor cells that would not be eliminated by the antibody alone ("crossfiring" or "bystander" effect) [2]. A number of other radioimmunoconjugates using these radioisotopes are also being evaluated for the treatment of NHL. There are other β^- emitting isotopes such as copper-67 (^{67}Cu), lutetium-177 (^{177}Lu), rhenium-186 (^{186}Re) and rhenium-188 (^{188}Re) which may be superior to ^{131}I and ^{90}Y for anti-tumor benefits, but their use is limited to very few centers [10].

Rhenium is the group II cogener of technetium. The coordination chemistry of rhenium is very similar (not identical) to that of technetium due to their periodic relationship. As a consequence, the bifunctional chelating systems (BFCs) and radiolabeling's methods developed for the ^{99m}Tc-labeling can be used for the ^{186}/^{188}Re-labeling of biomolecules [11]. ^{188}Re ($T_{1/2}$ = 16.9 h) is produced from beta-decay of the tungsten-188 father ($T_{1/2}$ = 69 d) through the elution of a ^{188}W/^{188}Re generator system. In addition to the emission of high energy electrons with average energy of 769 keV and beta emissions with energies of 2.12 MeV (71.6%) and 1.96 MeV (25.1%), ^{188}Re also decays with emission of gamma photon with energy of 155 keV (15%).

The aim of this work was to label the rituximab antibody with ^{188}Re using direct radiolabeling technique and show the radiochemical control of the optimized radiolabeling of liquid and lyophilized formulations.

2. METHODOLOGY

2.1. Reduction of rituximab

Rituximab (10 mg, MabThera/Roche) was reduced by reaction with 2-mercaptoethanol (2-ME/Sigma) to generate free sulfhydryl groups. Reaction took place at room temperature for 30 min and the resulting solution was passed through a PD-10 column (Sephadex G-25M, Pharmacia) using phosphate buffered saline (PBS) (pH 7.4) purged with nitrogen as mobile phase and fractions of 1 mL were collected (10 fractions). The concentration of the reduced antibody was determined by optical density at 280 nm on a UV/visible spectrophotometer (Hitachi Instruments U-2010). The number of resulting free sulfhydryl groups was assayed with Ellman's reagent (Sigma). Briefly: 50 µL of the sample of reduced antibody was mixed with 50 µL of the solution containing 0.3 mg/mL of Ellman's reagent and diluted to 1 mL with 0.1 M phosphate buffer pH 8. The mixture was incubated at room temperature for 5 min and the coloration (yellow color) measured in a UV/visible spectrophotometer at 407 nm. The number of thiols was obtained by comparison with a standard curve obtained by the assay of a series of cysteine standards ranging from 0.0125 – 0.1 mM. The result was expressed as sulfhydryl groups per antibody molecule.

2.2. Labeling of rituximab with ^{188}Re by direct method

The studies were done with a liquid (solution) and lyophilized (kit) formulation of the reduced rituximab that contained: 1 mg reduced rituximab; 82.8 mg sodium tartrate; 1 mg $SnCl_2$ and 0.25 mg gentisic acid. Perrhenate ($Na^{188}ReO_4$) eluted from the $^{188}W/^{188}Re$ generator (Polatom) in 0.9% NaCl was added (336.7 – 806.6 MBq). The mixture was then incubated for 1 hour at room temperature. For the liquid formulation the pH variation was studied and for this purpose the perrhenate solution was acidified with 3 drops of 12 M HCl. The conditions for lyophilization were: volumes of 1 mL of liquid formulation were filtered by Millipore (0.22 µm), delivered onto penicillin vials and then frozen and freeze-drying during 24 h. After this process, the kits were kept in a refrigerator.

2.3. Radiochemical control of ^{188}Re-rituximab

The labeling efficiency was evaluated by instant thin-layer chromatography (ITLC, Gelman Science Inc.). The paper was cut into 1.5 x 10 cm strips and activated by heating for 30 min, at 110°C as recommended by manufacturer's instructions. Both 0.9% NaCl and acetone were used as the mobile phases to separate free perrhenate ($^{188}ReO_4^-$). ^{188}Re-tartrate and $^{188}ReO_4^-$ moved with the solvent front (Rf = 1) when 0.9% NaCl was used, whereas radiocolloid ($^{188}ReO_2$) and ^{188}Re-rituximab remained at the origin (Rf = 0). In acetone, ^{188}Re-rituximab,

^{188}Re-tartrate and $^{188}ReO_2$ stayed at the origin and $^{188}ReO_4^-$ moved to the solvent front. Human serum albumin (5%)-impregnated ITLC-SG strips were used as the stationary phase and ethanol:ammonia:water (2:1:5 [v/v]) as the mobile phase to separate $^{188}ReO_2$ that remained at the origin while the ^{188}Re-rituximab, ^{188}Re-tartrate and $^{188}ReO_4^-$ moved with the solvent front.

3. RESULTS AND DISCUSSION

The average recovery of the protein was 99.3 ± 1.2% (n = 3). An excellent linear correlation was found between the cysteine concentration and absorbance (r = 0.9986). The number of SH groups generated per molecule of antibody was calculated based on the cysteine standard curve and after reduction with 2-ME was 12.0 ± 3.0 (average ± SD, n = 6).

The direct method of labeling consisted of the labeling solution containing rituximab; sodium tartrate, stannous chloride and gentisic acid reacting with the perrhenate solution at room temperature. To achieve the optimization of the radiolabeling yield, some variables were studied such as antibody mass, reducing agent mass, tartrate mass, reaction time, stability with the passing time, ^{188}Re volume and high activity. The results were presented in a previous work [12].

Table 1 summarizes the results of the best liquid formulation of ^{188}Re-rituximab labeling from this work.

Table 1. Results of radiolabeling using ^{188}Re

Standard radiolabeling (solution)
1 mg rituximab
82.8 mg tartrate
1.0 mg stannous chloride ($SnCl_2$)
0.25 mg gentisic acid
1 mL $^{188}ReO_4^-$
pH = 5.0
1 h reaction time (RT)
70 – 80% radiochemical purities
Stable up to 4 hours

The weak chelating agent (tartrate) was used to stabilize the reducing agent (stannous chloride) and the radioisotope (^{188}Re) in its intermediate oxidation state but it was noticed that sometimes tartrate was a competitor in the labeling reaction reducing the antibody labeling yield. Rhenium colloid was the biggest impurity in all the radiolabeling experiments.

Ferro-Flores *et al*. [13] has shown that the radiochemical purity was influenced by the pH and then, acid conditions were necessary to allow the reversible reaction of reduction of

Re^{7+}/Re^{5+}. Based on this fact some radiolabelings were done with ^{188}Re solution acidified with 3 drops of 12 M HCl and the results are shown in table 2.

Table 2. Influence of pH in the radiolabeling yield of ^{188}Re-rituximab

Experiment	pH	Radiolabeling yield (%)
1	1.0	34.5
2	3.5	66.7
3	4.0	73.0
4	5.0	79.0

It is known that the best labeling yields in direct protein labeling with ^{188}Re are achieved within a relatively narrow pH range: 4-5.5. Reaction at pH lower than 4 compromises the immunoreactivity of the antibodies, and higher pH contributes to reoxidation of $^{188}Re(V)$ back to perrhenate. In this study a pH value of 5.0 demonstrated its advantage over lower pH values in regards to the labeling yields.

The best formulation was also studied as a lyophilized formulation (kit). The kits were reconstituted with 1 mL of ^{188}Re (mean activity - 429.3 MBq), the reaction time was 1 h and took place at room temperature. The mean value of radiolabeling yield was 80.2 ± 8.8 % (average ± SD, n = 4). The results were approximately the same as the liquid formulation. Table 3 summarizes the results of kit formulation from this work compared with the results found in the literature [13].

Table 3. Comparison of the results found in this work and literature.

Lyophilized kit	Literature [13]
1 mg rituximab	2 mg rituximab
82.8 mg tartrate	87 mg tartrate
1.0 mg stannous chloride ($SnCl_2$)	2.3 mg stannous chloride ($SnCl_2$)
0.25 mg gentisic acid	0.5 mg gentisic acid
1 mL $^{188}ReO_4^-$	3 mL $^{188}ReO_4^-$
pH = 5.5	pH = 4.0
1 h reaction time (RT)	1 h reaction time (RT)
80% radiochemical purities	97% radiochemical purities
Unstable after 1 hour	Stable

It can be seen that the efficiency of rituximab labeling was considerably higher in the work from the literature than in this work and also the stability was better. In order to achieve different and better results another labeling route was chosen to perform more radiolabeling experiments using ^{188}Re and is currently being studied.

4. CONCLUSIONS

The reduction of the antibody anti-CD20 (rituximab) was efficient with high recovery of the reduced protein. Quality control methods for evaluation of radiochemical purity showed promising labeling yields of the antibody. Further studies will be carried out in order to check its *in vitro* and *in vivo* behavior and stability.

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