Original Article

Radioiodine D Amino Acids Labeling of Rituximab, A New Method for Enhancing the Radiopharmaceutical Targeting and Biostability

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ABSTRACT

Introduction: Radioimmunotherapy (RIT) is a very promising new therapy for the treatment of recurrent B-Cell non-Hodgkin's lymphoma (NHL). Iodine-131 is the most frequently used nuclide in clinical RIT, but its usefulness has been limited by dehalogenation of monoclonal antibodies labeled via conventional methods. To circumvent this problem, we have synthesized a tri-peptide consisting of non-metabolizable D amino acids attached to N-Hydroxysuccinimide (NHS).
Methods: Tri-peptide was synthesized by standard Fmoc solid phase synthesis on tritylchloride resin. Labeling of tri-peptide was performed using the chloramine-T method and the conventional extraction. Radioiodination of tri-peptide was followed by conjugation to anti-CD20 antibody. In vitro stability of labeled antibody in serum and phosphate buffered saline (PBS) was measured for 48hr by (thin layer chromatography) TLC. Raji cell line was used to test cell binding of the labeled anti-CD20.
Results: The chemical purity of synthesized peptide as assessed by analytical (high performance liquid chromatography) HPLC was 95%. Labeling of tri-peptide resulted in a radiochemical yield of 50-71% with radiochemical purity of > 95%. At Rituximab concentration of 10mg/ml, coupling efficiencies of 65-80% was obtained with radiochemical purity of 95% and Specific activity (SA) of 185MBq/mg (5mCi/mg).
Conclusion: This study showed that labeling monoclonal antibodies with radioiodine by non-metabolizable D amino acids will improve bio-stability of the product.

Key Words: Radioimmunotherapy, Dehalogenation, D amino acid, Iodine-131

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INTRODUCTION

The first antibody to be approved in 1997 for treating non-Hodgkin's lymphoma was Rituximab, a chimeric anti-CD20 immunoglobulin (1). Encouraging response rates of 30-50% have been reported in patients with relapse or refractory NHL treated with the unlabeled CD20 monoclonal antibody Rituximab, but complete remissions occur in only 5-10% of patients (2). Mechanisms of resistance to Rituximab and other unconjugated antibodies may involve inadequate serum antibody levels, insufficient binding of antibody due to lack of or loss of antigen expression on the lymphoma cells, poor access of antibody to the tumor cells, or failure of host effectors mechanisms to eliminate tumor cells despite antibody binding (3).

A rational approach to enhance the efficacy of anti-CD20 antibodies involves conjugating with cytotoxic radionuclides. Because of the inherent radiosensitivity of indolent lymphoma cells, radioimmunoconjugates have the ability to provide direct cytotoxicity even if, Antibody-dependent cell-mediated cytotoxicity (ADCC), a major mediator of rituximab activity impaired. Further more radioimmunotherapy with an anti-CD20 antibody conjugated to a beta-emitting radioisotope will deliver radiation not only to tumor cells that bind the antibody, but also due to a cross-fire effect, to neighboring tumor cells inaccessible to the antibody or with insufficient antigen expression (4).

CD20 antigen is expressed at high density on normal and malignant B cells and exhibits limited shedding, following antibody binding (1). The absence of antigen expression on stem cells allows for the recovery of normal B-cell following radioimmunotherapy which leads to the destruction of both malignant and normal B-cells.

various murine monoclonal antibodies have been approved for radioimmunotherapy of NHL, e.g. ^{90}Y-ibritumomab tiuxetan (Zevalin 2002) and ^{131}I-tositumomab (Bexxar) (5).As the treatment with murine antibody radiolabeled conjugates may induce human anti mouse antibodies (HAMA) and the fact that relapse of indolent NHL is probable despite the increasing efficacy of current therapies (6), we decided to radioiodinate the human/murine chimeric anti-CD20, Rituximab , to prevent induction of HAMA and to allow repeated administration.

Iodine-131 provides a relatively inexpensive and readily available radiopharmaceutical agent. It has a relatively long half life (8.1 days) with beta emission of 0.69 Mev for therapy, and a gamma emission for imaging, which is useful for making dosimetry estimates(1). The principle disadvantage of using iodine-131 isotope is the long circulation time of radioiodinated antibodies in blood stream, which leads to high back-ground radiation. This problem is complicated with dehalogenation of antibodies labeled via conventional procedure. Such removal of radioiodine from target cells, reduce the residence time of radioiodine in target cells which significantly affects radioimmunotherapy effectiveness (7).

There has been an ongoing effort to develop new methods of radioiodination of labeled antibodies. Early work was based on the use of non-metabolizable carbohydrates as linking agents, dilactitol-tyramine(8) and tyramine cellobiose (9) are two substrates examined for this purpose. Another technique involves the use of non metabolizable peptide adduct (10).

In this study, we describe synthesize of NHS-tri-peptide containing D amino acids as a new labeling method for production of stable radioiodinated antibodies.

METHODS

All chemicals were obtained from commercial sources and used without further purification. Tritylchloride resin and 9-fluorenylmethoxy carbonyl (Fmoc)-protected amino acids were purchased from Nova Biochem (Germany). The reactive side chains of the amino acids were masked with one of the following groups: Lysine, t-butoxycarbonyl (Lys-Boc), Tyrosine, t-butyl (Tyr-tBu). 1-Hydroxy-benzothiazol (HOBT), diisopropylcarbodiimide (DIC), N-methylpyrolidone (NMP), piperidine, N, N-dimethlyformamide (DMF), dicyclohexylcarbodiimide (DCC) were purchased from Fluka. Rituximab (Mabthera), a mouse –human chimeric anti-CD20 antibody was commercially obtained from Roche (switzerland 100mg/10ml). Raji cell line, a Human Burkitt's lymphoma was purchased from the Pasteur Institute of Iran. Cell line was cultured in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum, Gentamycine (100units/ml) and L-glutamine (2mM) in humidified incubator at 37°C and 5% Co_2. Iodine-131 was prepared from radioisotope division (Atomic Energy Organization of Iran).

^{1}H NMR spectra were obtained with BRUKER (500 MHZ) instrument using $CDCl_3$ as solvents. Analytical HPLC was carried out on a JASCO 880-PU intelligent pump HPLC system (Tokyo, Japan). CC 250/4.6 Nucleosil 120-3 C18 columns from Macherey-Nagel (Germany) were used for analytical HPLC. The gradient systems consisted of 0.1% trifluoroacetic acid (TFA)/water (solvent A), acetonitrile (solvent B). For

analytical HPLC, this gradient was used: 0 min 95%A(5%B), 5 min 95%A(5%B), 30 min 0%A(100%B), 35 min 0%A(100%B), 40 min 95%A(5%B), flow: 1ml/min.

Synthesis of Tri-peptide-NHS:

The peptide was synthesized by standard Fmoc solid phase synthesis on trityl chloride resin (substitution, 0.8mmol/g) on a semiautomatic peptide synthesizer (11). The Fmoc protected first amino acid D-lysine, is attached via its carboxyl end to resin then the peptide is enlarged by sequential addition of amino acids. Coupling of each amino acid was performed in the presence of 3 molar excess of F-moc amino acid, 4.5 molar excess of HOBt, 3.3 molar excess of DIC and 8 molar excess of DIPEA in NMP for 1.5h. The reaction was monitored by the ninhydrine test for each step and the F-moc group was removed by treatment with 20% piperidine in DMF for 30min. After coupling the last D-tyrosine , the peptide was cleaved from the resin and collected by filtration of the reaction mixture, solvent removal furnished the protected peptide as a gummy material which was essentially pure as determined by analytical HPLC (>95%) and was used as such. Deprotected tri-peptide had a retention time of 14 minute (Figure 1).

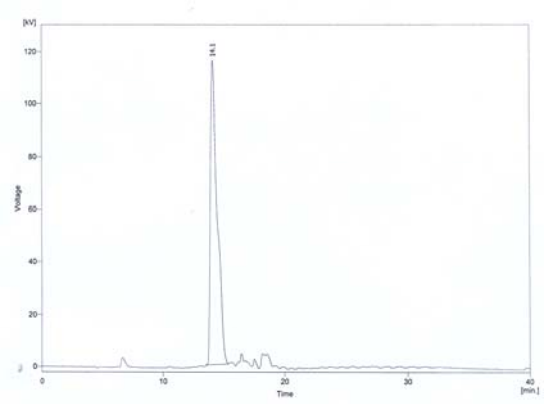

Figure 1: HPLC analysis of De-protected tri-peptide with retention time of 14 minute.

N-Hydroxysuccinimide was coupled to the C-terminal of tri-peptide by a solution of DCC in DMF with the same molar (12). It gives brown residue with a yield of 60% and a retention time of 32 minute on analytical HPLC (Figure 2). ^{1}H NMR: ($CHCl_3$) δ:1.31(t, 2H, *J*=4Hz); 1.39(s, 9H); 1.431(s, 18H); 1.596(t, 2H, *J*=3.5Hz); 1.725(t, 2H,*J*=3.5Hz); 2.705(s, 4H); 2.931(t, 2H,*J*=6Hz); 3.051(m, 4H, *J*=10.5Hz); 4.149(m, 1H); 4.163(t, 1H); 4.265(m, 2H); 4.314(t, 1H); 4.328(t, 1H), 6.912(dd, 4H, *J*=17.5Hz); 7.032(dd, 4H, *J*=10Hz); 7.295-7.544(m, 6H).

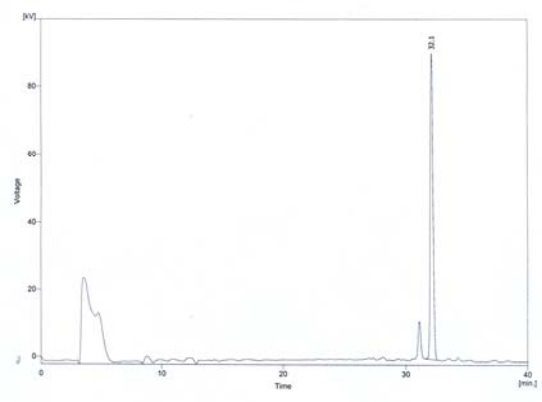

Figure 2: HPLC analysis of NHS-tri-peptide with retention time of 32 minute.

Preparation of [^{131}I] I-tri-peptide-NHS:

Tri-peptide-NHS (22mg, 0.024mmol) was dissolved in DMSO (2ml) and frozen in distributed tubes. 2µl (20µg, 2.2×10^{-5}) of tri-peptide-NHS was added to 50µl of labeling buffer: 0.5M Sodium phosphate buffer, pH=7.5, 200µCi (7.4MBq) $Na^{131}I$, followed by 25µl chloramine-T (4mg of chloramine-T in 1ml PBS 0.2M, pH=7.5). The components were mixed by pipette for 1 minute and the reaction was terminated by extracting the I^{131}-labeled tri-peptide-NHS through adding 100µl extraction solvent: Benzene/DMF (100/5). After mixing with a pipette, the organic phase was removed to a glass vial and dried the organic phase with a gentle stream of nitrogen. The extraction of the aqueous phase was repeated twice following the steps describe above. The extract fraction of [^{131}I] I-tri-peptide-NHS was analyzed by TLC.

Labeling Rituximab with [^{131}I] I-tri-peptide-NHS and $Na^{131}I$:

The solvent of [^{131}I] I-tri-peptide-NHS extract was completely removed with stream of nitrogen. 1µl of Rituximab (10µg, 6.6×10^{-5}µmol) was mixed with 100µl Borate Buffer pH=8.5 and incubated with [^{131}I] I-tri-peptide-NHS at room temperature for 2h. The labeled antibody was isolated by gel filtration column (Sephadex G-50 Pharmacia). The column was eluted with PBS buffer as the mobile phase. The effluent was collected in fraction of 1 ml. Radioactivity in each fraction was determined by γ-counter (EG & G,

ORTEC, Model 4001M), and the fraction containing the labeled antibody was separated (Figure 3).

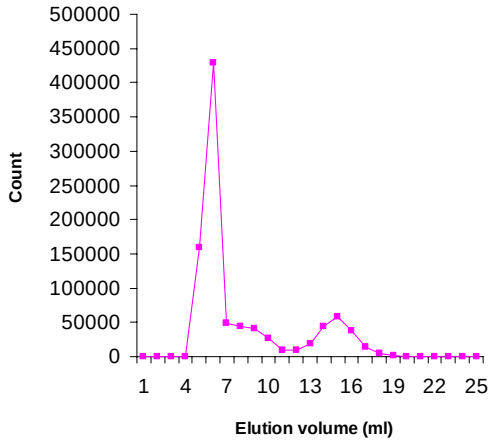

Figure 3: Size-exclusion chromatography of Rituximab labeled by [^{131}I] I-tri-peptide-NHS, The peaks at elution volume 6ml and 15ml correspond to γ-count of [^{131}I] I-tri-peptide-Rituximab and unconjugated [^{131}I] I-tri-peptide-NHS.

At the direct labeling method, 1µl of Rituximab (10µg, 6.6×10^{-5}µmol) was added to 50µl of sodium phosphate buffer (0.5M, pH=7.5), followed by 200µci $Na^{131}I$ (7.4MBq in 0.1N NaOH) and 25µl of chloramine-T (4mg/1ml PBS 50mM, pH=7.5). The mixture was stirred for 1 minute and 50µl of sodium metabisulfite (SMB: 4mg/1ml PBS 50mM, pH=7.5) followed by 100µl KI (10mg/1ml). The reaction mixture was immediately applied to a gel filtration column (Sephadex G-50 Pharmacia) and the fraction containing ^{131}I-rituximab was isolated as describe above.

Quality Control:

The radiochemical purity of [^{131}I] I-tri-peptide-NHS was assessed by instant thin-layer chromatography (ITLC) on silica gel 60 (TLC-SG). TLC-SG was performed using ethyl acetate-methanol (9:1 v/v) as mobile phase. The labeling efficiency was determined by ITLC on Whatman 3MM paper. The strip was placed in a solvent (NaCl, 0.9% w/v) that permit free iodine-131 to migrate with the solvent front while [^{131}I] I-tri-peptide- Rituximab remained at the origin. Then the strip was cut in half and each part was counted in a γ-counter.

Cell Binding Test:

In order to evaluate the effect of [^{131}I] I-tri-peptide-NHS on conjugation with Rituximab, the specificity of labeled Riruximab was measured with a direct cell binding assay. Raji cells are known for expression of high amount of CD20 antigen (1). Identical amount of antibody (10ng), labeled with 10 kBq iodine-131 were incubated at 4°C for 2h with 5 sequential dilution of Raji cells, between 10^6 and 10^7 cells in exponential growth, in a volume of 500µl of phosphate-buffered saline.

RESULTS

Synthesis and Labeling:

Tri-peptide-NHS was synthesized in an overall yield of about 60% with the purity of >95% as determined by Analytical HPLC. Labeling of tri-peptide was performed using the chloramine-T method and the conventional extraction, resulting in a radiochemical yield of 50-71% and radiochemical purity of > 95%. Radioiodination of tri-peptide was followed by conjugation to Rituximab antibody with 65-80% labeling efficiency and 95% radiochemical purity as shown in Figure 4.

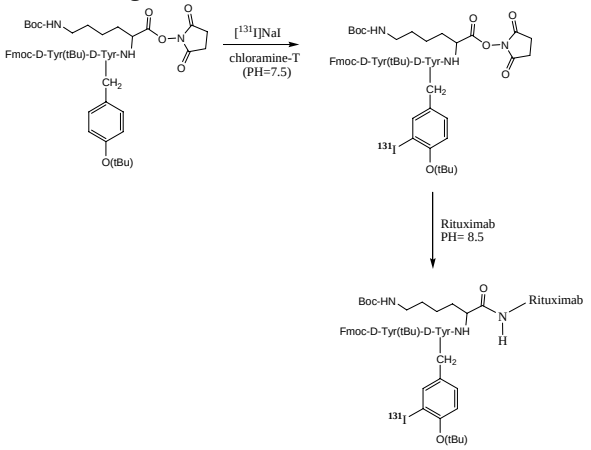

Figure 4. Preparation of [^{131}I] I-tri-peptide-NHS and labeling of Rituximab.

Quality Control:

The extract fraction of [^{131}I] I-tri-peptide-NHS was analyzed by TLC-SG using ethyl acetate-methanol (9:1 v/v) as mobile phase resulting in Rf=0.82 for [^{131}I]I-tri-peptide-NHS and Rf=0.16 for tri-peptide-NHS. Stability of labeled antibody was assessed in the presence of PBS solution and human serum up to 48h at 37°C. Data in table 1 shows that stability of our labeled compound in human serum in the presence of deiodinaze enzyme was comparable with results

obtained in PBS without enzyme. For rituximab labeled with direct method a considerable decrease of stability in human serum media has been obtained (Table 2). The serum stability of two methods compared as shown in Figure 5.

Table 1: The stability of the [^{131}I] I-tri-peptide-Rituximab as measured by TLC in two media (PBS, Serum) up to 48h.

Time post labeling	PBS	Serum media
4h	87%±1.5	85%±2.0
24h	78%±0.5	75%±1.2
48h	73%±2.0	71%±1.0

Table 2: The stability of the [^{131}I] I-Rituximab as measured by TLC in two media (PBS, Serum) up to 48h.

Time post labeling	PBS	Serum media
4h	92%±1.2	79%±1.0
24h	82%±2.0	65%±1.2
48h	79%±1.5	58%±2.0

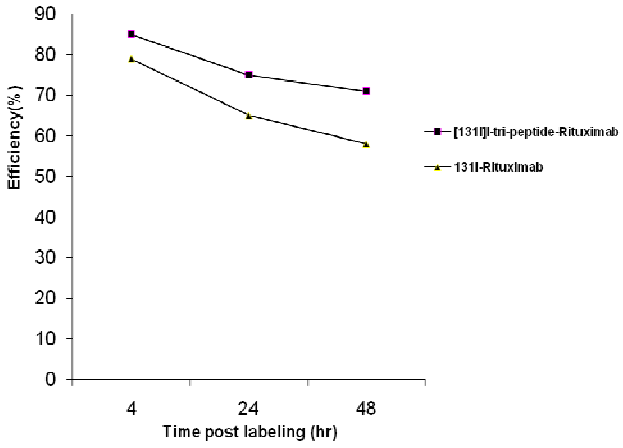

Figure 5: The serum stability of [^{131}I]I-tri-peptide-Rituximab and [^{131}I]I-Rituximab for 48 hr at 37°C.

Cell Binding Test:

Radioiodine must be attached to the targeting antibody to provide a stable conjugate without compromising the affinity and specificity of the antibody for the target antigen. The result showed that labeled [^{131}I] I-tri-peptide- Rituximab preserves its specificity and avidity more than direct method ([^{131}I] I - Rituximab (Figure 6).

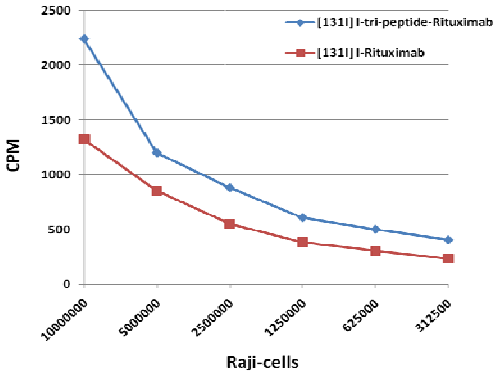

Figure 6: The Cell Binding of [^{131}I]I-tri-peptide-Rituximab and [^{131}I]I-Rituximab measured by direct radioimmunoassay. The results are expressed in Count per Minute of radiolabel Rituximab bound to Raji cells.

DISCUSSION

The iodine-131 labeled Fmoc-D-Tyr(t-Bu)-D-Tyr(t-Bu)-Boc-D-lys-NHS was chosen for this study. These amino acids were connected via non- metabolizable amide bonds. To improve radioactivity residualization, we designed hydrophilic tri-peptide with all protecting groups (Fmo, Boc, t-Bu) which have Molecular weight of more than 500 (8). This peptide increases the stability of radioiodine at the tumor site following Rituximab attachment to the cell surface antigen (13).

Labeling of this peptide was performed by electrophilic substitution of iodine-131 in D-tyrosine ring, using chloramine-T as oxidant. Since the NHS ester group in tri-peptide is sensitive to hydrolyze, the radioiodination was terminated after 1 minute by conventional extraction of labeled peptide into Benzene/DMF and evaporated to dryness with a stream of nitrogen.

To provide a stable conjugate without affecting the affinity and specificity of antibody for target antigen (CD20), Rituximab was added to the labeled peptide in buffer media (Borate pH=8.5) and permitted to react in room temperature for 2h. Differential protections of γ amine of D-lysine and the peptide' N-terminus with Fmoc and Boc protecting groups, enable specific conjugation of activated [^{131}I] I-tri-peptide-NHS to NH_2-lysine antibody (Figure 4). The labeled Rituximab was isolated, using a 1×20-cm Sephadex G-50 column. An important consideration in this

method is that in our condition reaction, we can prevent denaturation of the monoclonal antibody.

The in vitro stability of the rodioiodinated antibody under physiological condition is a very important parameter in the evaluation of de-halogenation which gives free iodide and causes radiation dose exposure of non-target tissues and limiting the clinical efficacy. Thus stability of labeled antibody was assessed in the presence of PBS solution and human serum up to 48h at 37°C. Studies have demonstrated that labeled antibody is radiochemically stable in both BSA and human serum for 2 days in comparison with direct method .

CONCLUSION

Our study introduced a new tri-peptide for labeling monoclonal antibodies with I-131. This new labeled antibodies are more radiostable and has potential effect in treatment of patients with lymphoma.

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