Published in final edited form as:

Nucl Med Biol. 2013 July ; 40(5): 600–605. doi:10.1016/j.nucmedbio.2013.01.012.

Preclinical Evaluation of NETA-Based Bifunctional Ligand for Radioimmunotherapy Applications using ^{212}Bi and ^{213}Bi : Radiolabeling, Serum Stability, and Biodistribution and Tumor Uptake Studies

Chi Soo Kang¹, Hyun A Song¹, Diane E. Milenic², Kwamena E. Baidoo², Martin W. Brechbiel², and Hyun-Soon Chong^{1,*}

¹Chemistry Division, Biological and Chemical Sciences Department, Illinois Institute of Technology, Chicago, IL

²Radioimmune & Inorganic Chemistry Section, Radiation Oncology Branch, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD, USA

Abstract

Introduction—Despite the great potential of targeted α -radioimmunotherapy (RIT) as demonstrated by pre-clinical and clinical trials, limited progress has been made on the improvement of chelation chemistry for ^{212}Bi and ^{213}Bi . A new bifunctional ligand 3p-*C*-NETA was evaluated for targeted α RIT using ^{212}Bi and ^{213}Bi .

Methods—Radiolabeling of 3p-*C*-NETA with $^{205/6}\text{Bi}$, a surrogate of ^{212}Bi and ^{213}Bi , was evaluated at pH 5.5 and room temperature. *In vitro* stability of the $^{205/6}\text{Bi}$ -3p-*C*-NETA-trastuzumab conjugate was evaluated using human serum (pH 7, 37 °C). Immunoreactivity and specific activity of the $^{205/6}\text{Bi}$ -3p-*C*-NETA-trastuzumab conjugate was measured. An *in vivo* biodistribution study was performed to evaluate the *in vivo* stability and tumor targeting properties of the $^{205/6}\text{Bi}$ -3p-*C*-NETA-trastuzumab conjugate in athymic mice bearing subcutaneous LS174T tumor xenografts.

Result—The 3p-*C*-NETA-trastuzumab conjugate was extremely rapid in complexing with $^{205/6}\text{Bi}$, and the corresponding $^{205/6}\text{Bi}$ -3p-*C*-NETA-trastuzumab was stable in human serum. $^{205/6}\text{Bi}$ -3p-*C*-NETA-trastuzumab was prepared with a high specific activity and retained immunoreactivity. $^{205/6}\text{Bi}$ -3p-*C*-NETA-trastuzumab conjugate displayed excellent *in vivo* stability and targeting as evidenced by low normal organ and high tumor uptake.

Conclusion—The results of the *in vitro* and *in vivo* studies indicate that 3p-*C*-NETA is a promising chelator for RIT applications using ^{212}Bi and ^{213}Bi . Further detailed *in vivo* evaluations of 3p-*C*-NETA for targeted α RIT are warranted.

Keywords

Bi-212; Bi-213; bifunctional chelator; serum stability; biodistribution; radiolabeling

© 2013 Elsevier Inc. All rights reserved

*Corresponding Author: Hyun-Soon Chong Chemistry Division, Biological and Chemical Science Department Illinois Institute of Technology 3101 S. Dearborn St, LS 182 Chicago, IL, 60616. Chong@iit.edu Fax: 312-567-3494.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Radioimmunotherapy (RIT) using α -particle emitting radioisotopes has been proven to be effective in the treatment of blood-borne cancers including leukemia and lymphoma as well as micrometastatic cancers [1,2]. Radionuclides of Bi(III), ^{213}Bi ($t_{1/2} = 45$ m) and ^{212}Bi ($t_{1/2} = 60$ min) with high energies (5–8 MeV) and a short emission range (50–80 μm) have been proposed to effectively kill tumor cells with minimal damage imparted to normal tissues [1]. The therapeutic efficacy of ^{213}Bi has been demonstrated in pre-clinical and clinical trials including the landmark clinical trial [3] of patients with advanced myeloid leukemia at Memorial Sloan-Kettering Cancer Center [3–7]. Furthermore, ^{213}Bi was found to be effective in chemo- and external radiation-resistant leukemia as shown in pre-clinical studies [8]. Thus, it is already established that for leukemias and malignancies that are dispersed and also readily accessible, α -particle RIT is effective.

Despite the great potential of targeted α -RIT of cancers, limited progress has been made on improvement of chelation chemistry for ^{212}Bi and ^{213}Bi . Several bifunctional ligands including DOTA (1,4,7,10-Tetraazacyclododecane- N,N',N'',N''' -tetraacetic acid) and DTPA (diethylenetriamine pentaacetic acid) analogues (Figure 1) have been used for the production of ^{212}Bi or ^{213}Bi -labeled radioimmunoconjugates [1]. Radiolabeling of an antibody conjugate with ^{212}Bi and ^{213}Bi should be optimized under mild conditions to avoid radiolytic damage of the antibody. Given the short half-life of ^{212}Bi and ^{213}Bi , the slow complexation kinetics of DOTA with Bi(III) under mild conditions remains an unacceptable problem for RIT applications of this ligand with Bi(III) radionuclides [1, 9–10]. In general, the DTPA derivatives display fast complexation kinetics with Bi(III) as compared to DOTA, however, Bi(III)-DTPA complexes are not as stable as Bi(III)-DOTA [1,11–12]. More effective bifunctional ligands are needed for practical and larger scale production of stable ^{213}Bi or ^{212}Bi -labeled radioimmunoconjugates under the mild radiolabeling conditions required for proteins. Development of an effective chelator that can rapidly and tightly bind to ^{212}Bi or ^{213}Bi is expected to significantly contribute to active clinical exploration of the α -emitting radioisotopes for targeted RIT applications of various cancers using different antibodies, their fragments and other delivery vectors that suitably match biological and radionuclide half-lives. We developed an octadentate chelator NETA ({4-[2-(bis-carboxymethyl-amino)-ethyl]-7-carboxymethyl-[1,4,7]triazonan-1-yl}-acetic acid) for RIT applications [13–14]. NETA was proposed to complex Bi(III) with enhanced complexation kinetics and stability via bimodal and cooperative binding using both acyclic and macrocyclic moieties. The *in vitro* and *in vivo* complexation studies indicate that NETA was found to be effective in sequestering Bi(III), and the corresponding Bi(III)-NETA complex was stable in human serum and mice [15]. We herein report preclinical evaluation of a NETA-based bifunctional ligand 3p-C-NETA for targeted α RIT applications with ^{212}Bi and ^{213}Bi . The new bifunctional chelator 3p-C-NETA was investigated for its effectiveness in sequestering Bi(III) radionuclides for potential use in RIT applications. 3p-C-NETA was conjugated to a tumor targeting antibody, trastuzumab, and the corresponding 3p-C-NETA-trastuzumab was evaluated for radiolabeling efficiency and specific activity with Bi(III). The Bi(III) radiolabeled 3p-C-NETA-trastuzumab conjugate was further evaluated for *in vitro* serum stability, immunoreactivity, and biodistribution and tumor uptake using LS-174T tumor bearing mice.

2. Material and methods

2.1. Reagents and Analyses

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) and used as received unless otherwise noted. Trastuzumab (Herceptin; Genetech, South San Francisco, CA) was obtained through the Veterinary Resources Program (National Institutes of Health,

Bethesda, MD). $^{205/6}\text{Bi}$ was produced using a CS30 cyclotron (PET Dept, Clinical Center, NIH) and purified as previously described [4]. Size-exclusion HPLC (SE-HPLC) chromatograms were obtained on an Agilent 1200 equipped with a diode array detector and an in-line IN/US γ -Ram Model 2 radiodetector (Tampa, FL), fitted with Bio-Silect SEC 250-5 column (Biorad, Hercules, CA) or a TSKgel G3000PW column (Tosoh Biosep, Montgomeryville, PA).

2.2. Conjugation of 3p-C-NETA to Trastuzumab

3p-C-NETA [16] was prepared as previously reported and conjugated to trastuzumab, and the corresponding trastuzumab conjugates were characterized by the spectroscopic methods as described in our previous publications [9,17].

2.3. Radiolabeling of bifunctional ligand-trastuzumab conjugates with $^{205/6}\text{Bi}$

All HCl solutions were prepared from ultra-pure HCl (Fisher, Cat# A466-500). For metal-free radiolabeling, plastic ware including pipette tips, tubes, and caps were soaked in 0.1M HCl overnight, washed thoroughly with Milli-Q (18.2 M Ω) water, and air-dried overnight. Ultra pure NH_4OAc (Aldrich, #372331) was used to prepare buffer solutions (0.1M NH_4OAc and 5M NH_4OAc , pH 7) which were treated with Chelex-100 resin (Biorad, #142-2842, 1g/100 ml buffer solution), shaken overnight at room temperature, and filtered through 0.22 μm filter (Corning, #430320) prior to use. To a buffer solution (120 μL / 0.1 M for 3p-C-NETA-trastuzumab, pH 7) in a capped microcentrifuge tube (1.5 mL, Fisher Scientific #05-408-129) was sequentially added a solution of 3p-C-NETA-trastuzumab (30 μg) in PBS (7.7 μL) and $^{205/6}\text{Bi}$ (0.1 M HI, 60.5 μCi , 12.7 μL). The final volume of the resulting solution (pH 5.5) was 140.4 μL . The reaction mixture was agitated on a thermomixer (Eppendorf, #022670549) at 1,000 rpm at room temperature for 1 h. The labeling efficiency was determined by SE-HPLC (Biorad, Bio-Silect SEC 250–5 column, 7.8 $\times$ 30 cm, eluent: PBS, flow rate: 1 mL/min). A solution of the radiolabeling reaction mixture (10 μL) was withdrawn at the designated time points (1, 5, 10, 20, 30, and 60 min), and DTPA solution (10mM, 1 μL) was added to the mixture, and the resulting mixture was incubated at room temperature for at least 20 min. Samples were evaluated by SE-HPLC. Peaks for bound and unbound radioisotope appeared around 8.3 min and 11 min, respectively.

2.4. In vitro stability of $^{205/6}\text{Bi}$ -3p-C-NETA-trastuzumab conjugate

$^{205/6}\text{Bi}$ -3p-C-NETA-trastuzumab was prepared by reaction of 3p-C-NETA-trastuzumab with $^{205/6}\text{Bi}$ at RT (0.1 M NH_4OAc , pH 5.5). The complex formed was purified from $^{205/6}\text{Bi}$ by gel filtration chromatography using PD-10 column (GE Healthcare, Cat# 17-0851-01) eluted with PBS (pH 7.4, Fisher Scientific, Cat# BP2438-4). The fractions containing $^{205/6}\text{Bi}$ -3p-C-NETA-trastuzumab (320 μL , 30 μCi / 320 μL , 50 μCi / 470 μL , 10 μCi for triplicate samples) were combined and added into human serum (320 / 320 / 470 μL respectively, Gemini Bioproducts, #100110) in a microcentrifuge tube (Fisher Scientific, #05-408-129). The stability of the purified $^{205/6}\text{Bi}$ -3p-C-NETA-trastuzumab was evaluated for 7 days using SE-HPLC as described above. A solution of the radiolabeled complex in serum (15–80 μL) was withdrawn at the designated time point, treated with DTPA (5 mM, 1.4 μL), incubated at room temperature for 20 min and then diluted with PBS (pH 7.4, 100–200 μL) prior to SE-HPLC.

2.5. Preparation of $^{205/6}\text{Bi}$ -3p-C-NETA-trastuzumab for immunoreactivity and in vivo studies

For use in the *in vitro* binding and *in vivo* biodistribution and tumor targeting studies, $^{205/6}\text{Bi}$ -3p-C-NETA-trastuzumab with high specific activity was prepared. In this

instance, two aliquots of $^{205/6}\text{Bi}$ solution (100 μL in 0.1 M HI, 1.1 mCi each) were each neutralized to pH 5.5 using 5M NH_4OAc solution (10 μL , pH 7). An aliquot of 3p-C-NETA-trastuzumab containing 50 μg of protein in PBS was added to one or the other $^{205/6}\text{Bi}$ solution. The mixtures were vortexed briefly and incubated at 37°C. After 30 min, EDTA solution (5 μL , 0.1M) was added to each reaction, vortexed briefly, and further incubated for 5 min. The products were purified using disposable PD-10 columns. The labeling efficiency and the specific activity of the radiolabeled products were determined from the pooled fractions collected from the PD-10 columns. The purity of the labeled products as determined by SE-HPLC (TSKgel G3000PW column, PBS eluate at 0.5 mL/min) was >98%.

2.6. Radioimmunoassay

The immunoreactivity of the $^{205/6}\text{Bi}$ -labeled 3p-C-NETA-trastuzumab conjugate was assessed in a radioimmunoassay using purified recombinant human Erb2/Fc chimera (rhErb2/Fc, R&D systems). Briefly, 50 ng of the rhErb2/Fc in PBS with Mg^{+2} and Ca^{+2} (50 μL) was added to each well of a 96-well plate and allowed to adsorb to the well at 4 °C. Following an overnight incubation, the solution was removed and 100 μL of 1% BSA in PBS (BSA/PBS) was added to each well and incubated at room temperature for 30–60 min. Serial dilutions of $^{205/6}\text{Bi}$ -3p-C-NETA-trastuzumab (~300,000 to ~12,500 cpm in 50 μL of BSA/PBS) were added to the wells in duplicate, covered and incubated at 37 °C for 4 h. The radiolabeled antibody was then removed and the wells were washed three times with PBS. The radioactivity was removed from the wells with a solution of 0.2 M NaOH with 1 mM EDTA (100 μL), adsorbing the solution to cotton filters and transferring the filters to 12 $\times$ 75 mm polypropylene tubes. The radioactivity was measured in a γ -scintillation counter (WizardOne, Perkin Elmer, Shelton, CT), and the percentage binding calculated for each dilution. The values presented are an average of the percent bound for the serial dilutions. The specificity of the radiolabeled trastuzumab was confirmed by incubating one set of wells with radiolabeled trastuzumab and 10 μg of unlabeled trastuzumab.

2.7. In vivo biodistribution and tumor targeting studies

In vivo studies of $^{205/6}\text{Bi}$ -3p-C-NETA-trastuzumab conjugate were conducted using the human colon carcinoma cell line, LS-174T, (kindly provided by Dr. J. Greiner, NCI). The LS-174T cell line was grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10 mM glutamine along with 10% FetalPlex (Gemini Bio-Products, Woodland, CA) and 1 mM non-essential amino acids. Media and supplements were obtained from Lonza (Walkersville, MD). The cells were harvested for *in vivo* studies when they reached no more than 75% confluency. All *in vivo* studies were performed using 4–6 week old female athymic (nu/nu) mice (Charles River Laboratories, Wilmington, MA). All animal protocols were approved by the National Cancer Institute Animal Care and Use Committee. Mice were injected subcutaneously (s.c.) in the right rear leg with 2×10^6 LS-174T cells in media (200 μL) with 20% Matrigel (BD Biosciences, San Jose, CA). At the time of tumor cell implantation, cells were >95% viable. Mice were utilized in studies when the tumor xenografts maximal diameter measured 0.2 – 0.4 cm. Mice (n = 4 per time point) were injected intravenously (i.v.) with the $^{205/6}\text{Bi}$ -labeled trastuzumab conjugates (~7.5 μCi on 0.6 μg) and sacrificed by exsanguination at 2, 6 and 24 h. The blood, tumor, and major organs were collected, wet-weighted, and counted in a γ -scintillation counter. Tumor weights were 113.0 ± 32.0 , 97.8 ± 29.8 and 149.5 ± 66.6 at the 2, 6 and 24 h time points, respectively. The percent injected dose per gram (%ID/g) and standard deviation were calculated.

2.8. Statistical Analysis

The data of biodistribution and radiolabeling kinetics were analysed by Student's *t* test using Prism 3.0 software (GraphPad). Difference was considered significant if *p* values are less than 0.05.

3. Result and Discussion

3.1. Conjugation of bifunctional ligands to trastuzumab and radiolabeling of the corresponding conjugates with $^{205/6}\text{Bi}$

3p-*C*-NETA was prepared and conjugated to a tumor targeting antibody, trastuzumab. Trastuzumab was approved by the FDA for the treatment of metastatic breast cancer and is known to selectively target the HER2 protein overproduced in various tumors including colon and breast cancers [18–19]. The corresponding 3p-*C*-NETA-trastuzumab conjugate was purified as previously reported [9,16]. The 3p-*C*-NETA-trastuzumab was radiolabeled with $^{205/6}\text{Bi}$ ($t_{1/2} = 15.3$ d for ^{205}Bi ; $t_{1/2} = 6.2$ d for ^{206}Bi), a γ -emitting surrogate of ^{212}Bi and ^{213}Bi . It should be noted that it is not feasible to evaluate stability of the radiolabeled complex over a long period of time using ^{213}Bi and ^{212}Bi with the short half-life. $^{205/6}\text{Bi}$ has a significantly longer half-life and is much less costly relative to ^{213}Bi and ^{212}Bi . The Bi(III) radionuclides, $^{205/6}\text{Bi}$, ^{212}Bi , and ^{213}Bi have the same ionic radius (103 ppm) and prevalent oxidation state (+3) [1]. $^{205/6}\text{Bi}$ is well established as the standard radionuclide for evaluating complex stability and kinetics of bifunctional chelating agents for their use in sequestering Bi (III) radionuclides [9,15,20].

The purified 3p-*C*-NETA-trastuzumab conjugate (30 μg) in 0.1M NH_4OAc buffer solution at pH 5.5 was radiolabeled with $^{205/6}\text{Bi}$ (0.1M HI) (60.5 μCi) at room temperature (RT). Over a 1 h study period, the radiolabeling kinetics was determined by taking aliquots of the reaction mixture at 6 time points (1 min, 5 min, 10 min, 20 min, 30 min, and 60 min). The components were analyzed using SE-HPLC after quenching the reaction with 10 mM DTPA, and the radiolabeling efficiency (%) was determined (Table 1 and the Supporting Information). The data indicate that the $^{205/6}\text{Bi}$ -radiolabeling of 3p-*C*-NETA-trastuzumab conjugate were extremely rapid and nearly complete within 1 min ($93.4 \pm 1.9\%$, room temperature). 3p-*C*-NETA bound $^{205/6}\text{Bi}$ at a significantly greater rate relative to *C*-DOTA over the time points ($p < 0.0001$ at 1, 5, 10, 20 min, $p < 0.001$ at 30 min, and $p < 0.01$ at 60 min, Table 1). 3p-*C*-NETA-trastuzumab conjugate displayed radiolabeling kinetics with $^{205/6}\text{Bi}$ comparable to that of *C*-DTPA-trastuzumab [20].

3.2. In vitro serum stability of $^{205/6}\text{Bi}$ -3p-*C*-NETA-trastuzumab conjugate

The $^{205/6}\text{Bi}$ -3p-*C*-NETA-trastuzumab conjugate was further evaluated for complex stability using human serum. The $^{205/6}\text{Bi}$ -3p-*C*-NETA-trastuzumab was freshly prepared at room temperature, purified, and incubated in human serum at 37 °C over 1 week. At each time point, aliquots of the reaction mixture were analyzed using SE-HPLC after the addition of 10mM DTPA to react with any released Bi. $^{205/6}\text{Bi}$ -3p-*C*-NETA-trastuzumab was very stable in human serum without release of the radioactivity for at least 7 d (Figure 2 and Supporting Information). $^{205/6}\text{Bi}$ -3p-*C*-NETA-trastuzumab conjugate was favorably compared to $^{205/6}\text{Bi}$ -*C*-DOTA-trastuzumab conjugate and more stable than $^{205/6}\text{Bi}$ -*C*-DTPA-trastuzumab as previously reported [9,20].

3.3. Specific activity and immunoreactivity of $^{205/6}\text{Bi}$ -labeled trastuzumab conjugates

$^{205/6}\text{Bi}$ -3p-*C*-NETA-trastuzumab was evaluated for specific activity and immunoreactivity evaluations prior to performing comparative *in vivo* biodistribution studies. The 3p-*C*-NETA-trastuzumab was radiolabeled with $^{205/6}\text{Bi}$ with high specific activity (21.5 mCi/mg) and radiolabeling yield (84.8%). Immunoreactivity of the $^{205/6}\text{Bi}$ -radiolabeled trastuzumab

conjugates was assessed in a radioimmunoassay using recombinant human chimeric ErbB2/Fc. The specific binding of $^{205/6}\text{Bi-3p-C-NETA-trastuzumab}$ was 77.1%. Specificity was demonstrated by the addition of excess trastuzumab, with only 6.1% bound of the $^{205/6}\text{Bi-3p-C-NETA-trastuzumab}$.

3.4. In vivo biodistribution of $^{205/6}\text{Bi-3p-C-NETA-trastuzumab}$ conjugate

The *in vivo* stability and tumor targeting of $^{205/6}\text{Bi-3p-C-NETA-trastuzumab}$ conjugate was evaluated using athymic mice ($n = 4$) bearing subcutaneous LS174T tumor xenografts. We have successfully employed LS-174T cell line as a model for biodistribution and RIT studies using trastuzumab and other mAbs and previously reported that trastuzumab conjugates efficiently target the LS-174T xenografts [4,9,20–22]. Following i.v. injection of the $^{205/6}\text{Bi-3p-C-NETA-trastuzumab}$, the mice were euthanized at 2 h, 6 h, and 24 h. Tumors, selected organs, and the blood were harvested, wet weighed, and the radioactivity measured in a γ -counter (Figure 3 and Supporting Information). $^{205/6}\text{Bi-3p-C-NETA-trastuzumab}$ displayed increased tumor uptake over the time points ($10.80 \pm 2.2\%$ at 2 h; $12.82 \pm 3.0\%$ at 6 h; $24.10 \pm 4.8\%$ at 24 h). A significantly higher tumor uptake was noted at 24 h compared to other time points ($p < 0.01$). The radioactivity level of the blood at 2 h, 6 h, and 24 h post injection was $21.89 \pm 5.9\%$ (2 h), $20.78 \pm 5.5\%$ (6 h), and $9.92 \pm 2.2\%$ (24 h). A pronounced decrease in the radioactivity level in the blood was observed from 2 h to 24 h ($p < 0.01$). The $^{205/6}\text{Bi-3p-C-NETA-trastuzumab}$ exhibited a low tissue to blood ratio (< 0.7) in all tissues; a higher level of radioactivity was found in the tumor as compared to the normal organs over the study period. The tumor-to-blood ratio of the $^{205/6}\text{Bi-3p-C-NETA}$ was 2.50 at 24 h. Renal and liver uptake of $^{205/6}\text{Bi-3p-C-NETA}$ peaked at 6 h and decreased by 24 h ($7.59 \pm 1.9\%$ and $8.95 \pm 2.1\%$ at 6 h; $4.94 \pm 1.2\%$ and $5.60 \pm 2.4\%$ at 24 h, respectively). Femur uptake of $^{205/6}\text{Bi-3p-C-NETA}$ peaked at 2 h and then decreased over the time points ($2.91 \pm 2.6\%$ at 2 h; $2.58 \pm 0.8\%$ at 6 h; $2.51 \pm 0.5\%$ at 24 h). The *in vivo* biodistribution data indicate that $^{205/6}\text{Bi-3p-C-NETA}$ conjugated to trastuzumab remains stable in mice.

4. Conclusion

The octadentate ligand 3p-C-NETA conjugated to trastuzumab was extremely rapid in complexing $^{205/6}\text{Bi}$ ($> 93\%$ radiolabeling efficiency, 1 min), and the corresponding $^{205/6}\text{Bi-3p-C-NETA-trastuzumab}$ conjugate remained stable in human serum without releasing the radioactivity for 7 d. $^{205/6}\text{Bi-3p-C-NETA-trastuzumab}$ produced an excellent *in vivo* bioistribution profile and displayed excellent tumor uptake with a concomitant decrease of radioactivity in the blood with time. *In vitro* and *in vivo* experimental results indicate that 3p-C-NETA was shown to effectively sequester Bi(III) radionuclides. The new bifunctional chelator 3p-C-NETA with superior chelation chemistry with Bi(III) can be conjugated to various antibodies or peptides for targeted α RIT using ^{212}Bi and ^{213}Bi .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge financial support from the National Institutes of Health (R01CA112503). This work was partly supported by intramural research program of NIH.

Financial Support: Supported by National Institutes of Health (R01CA112503) and NIH intramural research program.

References

- [1]. Hassfjell S, Brechbiel MW. The development of the α -particle emitting radionuclides ^{212}Bi and ^{213}Bi , and their decay chain related radionuclides for therapeutic applications. *Chem Rev.* 2001; 101:2019–36. [PubMed: 11710239]
- [2]. Milenic DE, Brady ED, Brechbiel MW. Antibody-targeted radiation cancer therapy. *Nature Rev.* 2004; 3:488–98.
- [3]. Jurcic JG, Larson SM, Sgouros G, McDevitt MR, Finn RD, Divgi CR, Ballangrud AM, Klaus KA, Ma D, Humm JL, Brechbiel MW, Molinet R, Scheinberg DA. Targeted α particle immunotherapy for myeloid leukemia. *Blood.* 2002; 100:1233–9. [PubMed: 12149203]
- [4]. Milenic DE, Garmestani K, Brady ED, Albert PS, Ma D, Abdulla A, Brechbiel MW. Targeting of HER2 Antigen for the Treatment of Disseminated Peritoneal Disease. *Clin Cancer Res.* 2004; 10:7834–41. [PubMed: 15585615]
- [5]. Sgouros G, Ballangrud AM, Jurcic JG, McDevitt MR, Humm JL, Erdi YE, Mehta BM, Finn RD, Larson SM, Scheinberg DA. Pharmacokinetics and dosimetry of an alpha-particle emitter labeled antibody: ^{213}Bi -HuM195 (anti-CD33) in patients with leukemia. *J Nucl Med.* 1999; 40:1935–46. [PubMed: 10565792]
- [6]. Allen BJ, Raja C, Rizvi S, Li Y, Tsui W, Graham P, Thompson JF, Reisfeld RA, Kearsley J, Morgenstern A, Apostolidis C. Intraleisional targeted alpha therapy for metastatic melanoma. *Cancer Biol Ther.* 2005; 4:1318–24. [PubMed: 16322682]
- [7]. Kneifel S, Cordier D, Good S, Ionescu MC, Ghaffari A, Hofer S, Kretzschmar M, Tolnay M, Apostolidis C, Waser B, Arnold M, Mueller-Brand J, Maecke HR, Reubi JC, Merlo A. Local targeting of malignant gliomas by the diffusible peptidic vector 1,4,7,10-tetraazacyclododecane-1-glutaric acid-4,7,10-triacetic acid-substance P. *Clin Cancer Res.* 2006; 12:3843–50. [PubMed: 16778112]
- [8]. Friesen C, Glatting G, Koop B, Schwarz K, Morgenstern A, Apostolidis C, Debatin KM, Reske SN. Breaking chemoresistance and radioresistance with [^{213}Bi]anti-CD45 antibodies in leukemia cells. *Cancer Res.* 2007; 67:1950–8. [PubMed: 17332322]
- [9]. Song HA, Kang CS, Baidoo KE, Milenic DE, Chen Y, Dai A, Brechbiel MW, Chong HS. Efficient Bifunctional Decadentate Ligand 3p-*C*-DEPA for Targeted Alpha Radioimmunotherapy Applications. *Bioconjugate Chem.* 2011; 22:1128–35.
- [10]. Kumar K, Magerstadt M, Gansow OA. Lead(II) and bismuth(III) complexes of the polyazacycloalkane-*N*-acetic Acids nota, dota, and tetra. *J. Chem. Soc. Chem. Commun.* 1989; 9:145–6.
- [11]. Milenic DE, Roselli M, Mirzadeh S, Pippin CG, Gansow OA, Colcher D, Brechbiel MW, Schlom J. *In vivo* evaluation of bismuth-labeled monoclonal antibody comparing DTPA-derived bifunctional chelates. *Cancer Biother Radiopharm.* 2001; 16:133–54. [PubMed: 11385960]
- [12]. Nikula KN, McDevitt MR, Finn RD, Wu C, Kozak RW, Garmestani K, Brechbiel MW, Curcio MJ, Pippin CG, Tiffany-Jones L, Geerlings MW Sr, Apostolidis C, Molinet R, Geerlings MW Jr, Gansow OA, Scheinberg DA. Alpha-emitting bismuth cyclohexylbenzyl DTPA constructs of recombinant humanized anti-CD33 antibodies: Pharmacokinetics, bioactivity, toxicity, and chemistry. *J Nucl Med.* 1999; 40:166–76. [PubMed: 9935073]
- [13]. Chong HS, Garmestani K, Ma D, Milenic DE, Overstreet T, Brechbiel MW. Synthesis and biological evaluation of novel macrocyclic ligands with pendent donor groups as potential yttrium chelators for radioimmunotherapy with improved complex formation kinetics. *J Med Chem.* 2002; 45:3458–64. [PubMed: 12139456]
- [14]. Chong HS, Song HA, Birch N, Le T, Lim SY, Ma X. Efficient Synthesis and evaluation of bimodal ligand NETA. *Bioorg Med Chem Lett.* 2008; 18:3436–9. [PubMed: 18445528]
- [15]. Chong HS, Milenic DE, Garmestani K, Brady ED, Arora H, Pfister C, Brechbiel MW. *In vitro* and *in vivo* evaluation of novel ligands for radioimmunotherapy. *Nucl Med Biol.* 2006; 33:459–67. [PubMed: 16720237]
- [16]. Kang CS, Sun X, Jia F, Song HA, Chen Y, Lewis M, Chong HS. Synthesis and preclinical evaluation of bifunctional ligands for improved chelation chemistry of ^{90}Y and ^{177}Lu for targeted radioimmunotherapy. *Bioconjugate Chem.* 2012; 23:1775–82.

- [17]. Brady ED, Chong HS, Milenic DE, Brechbiel MW. Development of a spectroscopy assay for bifunctional ligand-protein conjugates based on copper. *Nucl Med Biol.* 2004; 31:795–802. [PubMed: 15246371]
- [18]. Pietras RJ, Pegram MD, Finn RS, Maneval DA, Slamon DJ. Remission of human breast cancer xenografts on therapy with humanized monoclonal antibody to HER-2 receptor and DNA-reactive drugs. *Oncogene.* 1998; 17:2235–49. [PubMed: 9811454]
- [19]. Agus DB, Bunn PA Jr, Franklin W, Garcia M, Ozols RF. HER-2/neu as a therapeutic target in non-small cell lung cancer, prostate cancer, and ovarian cancer. *Semin Oncol.* 2000; 27:53–63. [PubMed: 11236029]
- [20]. Dadwal M, Kang CS, Song HA, Sun X, Dai A, Baidoo K, Brechbiel MW, Chong HS. Synthesis and evaluation of a bifunctional chelate for development of Bi(III)-labeled radioimmunoconjugates. *Bioorg Med Chem Lett.* 2011; 21:7513–15. [PubMed: 22047687]
- [21]. Garmestani K, Chappell LL, Dadachova E, Yordanov A, Ma D, Schlom J, Brechbiel MW. *In vivo* comparison of macrocyclic and acyclic ligands for radiolabeling of monoclonal antibodies with Lu-177 for radioimmunotherapeutic applications. *Nucl Med Biol.* 2002; 29:431–42. [PubMed: 12031878]
- [22]. Milenic DE, Chappell LL, Ma D, Milenic DE, Garmestani K, Venditto V, Beitzel MP, Brechbiel MW. Synthesis and evaluation of novel bifunctional chelating agents based on 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid for radiolabeling proteins. *Nucl Med Biol.* 2003; 30:581–595. [PubMed: 12900284]

Figure 1.
Bifunctional ligands in preclinical and clinical evaluation

Figure 2. HPLC profile of ^{205}Bi -3p-C-NEtA-trastuzumab conjugate in PBS (pH 7.4, RT) at 2 h (A) and in human serum (pH 7, 37 °C) at 10 h (B) and 44 h (D)

Figure 3. Biodistribution of $^{205/6}\text{Bi-3p-C-NETA}$ in athymic mice bearing subcutaneous LS-174T tumors.

Table 1

*Radiolabeling efficiency (%) of bifunctional ligand-trastuzumab conjugates with $^{205/6}\text{Bi}$ (pH 5.5, room temperature, SE-HPLC).

Time (min)	3p-C-NETA *	C-DOTA #
1	93.4 ± 1.9	8.4 ± 1.9
5	95.0 ± 0.1	17.2 ± 4.3
10	94.9 ± 0.9	28.7 ± 4.5
20	94.7 ± 0.9	38.0 ± 4.9
30	95.4 ± 0.5	49.7 ± 9.0
60	95.3 ± 0.5	60.2 ± 8.0

* Radiolabeling efficiency (mean ± standard deviation) was measured in triplicate.

The data were cited for comparison (ref. 9).