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Anti-EGFRvIII monoclonal antibody armed with ^{177}Lu : *in vivo* comparison of macrocyclic and acyclic ligands

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Abstract

Introduction—Monoclonal antibody (mAb) L8A4 binds specifically to the epidermal growth factor receptor variant III (EGFRvIII) that is present on gliomas but not normal tissues, and is internalized rapidly after receptor binding. Because of the short range of its β -emissions, labeling this mAb with ^{177}Lu would be an attractive approach for the treatment of residual tumor margins remaining after surgical debulking of brain tumors.

Materials and Methods—L8A4 mAb was labeled with ^{177}Lu using the acyclic ligands [(R)-2-Amino-3-(4-isothiocyanatophenyl)propyl]-trans-(S,S)-cyclohexane-1,2-diamine- pentaacetic acid (CHX-A"-DTPA) and 2-(4-Isothiocyanatobenzyl)-6-methyldiethylene- triaminepentaacetic acid (1B4M-DTPA), and the macrocyclic ligands S-2-(4- Isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-tetraacetic acid (C-DOTA) and α -(5-isothiocyanato-2-methoxyphenyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10- tetraacetic acid (MeO-DOTA). Paired-label tissue distribution experiments were performed in athymic mice bearing subcutaneous EGFRvIII-expressing U87.)EGFR glioma xenografts over a period of 1 to 8 days to directly compare ^{177}Lu -labeled L8A4 to L8A4 labeled with ^{125}I using *N*-succinimidyl 4-guanidinomethyl-3- [^{125}I]iodobenzoate ([^{125}I]SGMIB).

Results—Except with C-DOTA, tumor uptake for the ^{177}Lu -labeled mAb was significantly higher than the co-administered radioiodinated preparation; however, this was also the case for spleen, liver, bone and kidneys. Tumor:normal tissue ratios for ^{177}Lu -1B4M-DTPA-L8A4 and to an even greater extent, ^{177}Lu -MeO-DOTA-L8A4, were higher than those for [^{125}I]SGMIB-L8A4 in most other tissues.

Conclusions—Tumor and normal tissue distribution patterns for this anti-EGFRvIII mAb were dependent on the nature of the bifunctional chelate used for ^{177}Lu labeling. Optimal results were obtained with 1B4M-DTPA and MeO-DOTA, suggesting no clear advantage for acyclic vs. macrocyclic ligands for this application.

Keywords

Lu-177; radioimmunotherapy; MeO-DOTA; 1B4M-DTPA

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1. Introduction

Radiation and its utility in the destruction of malignant tissues has been recognized almost since its discovery [1]. Selective targeting is the paramount focus of the contemporary therapeutic application of radionuclides. This is achieved by chemically packaging the radionuclide in such a way that it can take advantage of tumor-specific characteristics, hopefully leading to localized radiation deposition within malignant cell populations with minimal exposure of healthy tissues [2,3]. Much of this effort has involved monoclonal antibodies (mAbs), with ^{131}I being the most frequently utilized radionuclide, due in part to its reasonable cost, the availability of well-established radioiodination protocols and emission of γ -rays that permit imaging to be performed [4-8]. More recently, radiometalation has emerged as an attractive alternative for mAb labeling, exemplified by the FDA approval of ^{90}Y -labeled ibritumomab tiuxetan (Zevalin $\rightarrow$) for the treatment of patients with lymphoma [9].

Although ^{90}Y remains attractive for some applications of targeted radiotherapy, its high energy β -particles are not well matched to the treatment of minimum residual disease, settings thought to be most conducive for this type of therapy. For this reason, ^{131}I has been the radionuclide of choice for treatment of residual tumor cells remaining after surgical resection of brain tumors [10], despite the fact that its 364-keV γ -ray is not ideal for imaging, can increase radiation dose to normal brain, and can prolong patient confinement. Among the radiometals emitting low-energy β -particles, there are at least two attractive alternatives – ^{67}Cu and ^{177}Lu . While both present excellent characteristics for targeted radiotherapy, ^{177}Lu offers a compelling practical advantage – availability on a routine basis. Furthermore, its half-life (6.7 days), $E_{\beta}(\text{max})$ of 497 keV and average range in tissue (1.8 mm) are similar to those of ^{131}I ; however, the γ -rays emitted by ^{177}Lu are considerably lower in energy (113, 208 keV) and abundance (7, 11%) compared with those of ^{131}I . The γ -ray emission characteristics of ^{177}Lu are more suitable for imaging and dosimetry determination; in addition, they simplify radiation protection for patients and personnel, and are more amenable for clinical use as an outpatient procedure.

A number of groups including our own have been investigating a variety of bifunctional chelates derived from the acyclic ligand DTPA and the macrocyclic ligand DOTA for labeling mAbs and peptides with ^{177}Lu . As with many other radiometals, the primary prerequisite is the formation of complex with high thermodynamic stability and kinetic inertness under physiological conditions [11,12] in order to minimize release and translocation of the free radiometal to the bone [13]. A second criterion is minimizing activity levels in organs such as the liver and spleen, which can be higher for mAbs labeled with ^{177}Lu compared with those labeled using ^{131}I [14,15]. Based on these considerations, in a study comparing the mAb fragment HuCC49 CH2 labeled with ^{177}Lu using three different chelating agents, CHX-A"-DTPA, and C-DOTA were reported to provide good stability and tissue distributions while PA-DOTA did not [16]. Our own work using both the murine and chimeric forms of the anti-tenascin mAb 81C6 demonstrated that the *in vivo* behavior of complexed ^{177}Lu was more favorable with the MeO-DOTA macrocycle than either the NHS-DOTA macrocycle or the acyclic 1B4M- DTPA ligand [17].

For mAbs that undergo internalization after receptor binding and translocation from the cell surface to lysosomes, there is an additional criterion that must be considered – maximizing trapping of radiolabeled catabolites after proteolysis of the labeled mAb. While there is considerable evidence to suggest that this objective generally can be more readily accomplished with radiometals than with radiohalogens, the best bifunctional chelate for this purpose remains to be ascertained. As a first step, we performed a series of *in vitro* internalization and cellular processing assays with L8A4, a mAb that reacts with the rapidly internalizing epidermal growth factor receptor variant III, and labeled with ^{177}Lu using the acyclic ligands CHX-A"-DTPA, pSCN-Bz-DTPA, 1B4M- DTPA and the macrocyclic ligands C-DOTA and MeO-DOTA

[18]. Chelate-dependent differences in the intracellular retention of ^{177}Lu were observed, MeO-DOTA providing maximum internalized counts at early time points and the three DTPA-based ligands offering the highest values at later time points.

In the current study, we have evaluated the tissue distribution of the L8A4 mAb labeled with ^{177}Lu using two acyclic and two macrocyclic ligands previously evaluated in the *in vitro* studies - CHX-A"-DTPA, C-DOTA, 1B4M-DTPA and MeO-DOTA (Figure 1). A series of four paired label studies were performed in athymic mice with subcutaneous EGFRvIII-expressing xenografts and injected with one of the ^{177}Lu -labeled L8A4-chelate conjugates as well as with L8A4 labeled using *N*-succinimidyl 4-guanidinomethyl-3- [^{125}I]iodobenzoate [19]. The goal of this study was to identify the best ligand for labeling L8A4 mAb with ^{177}Lu to move forward for clinical evaluation as a targeted radiotherapeutic for glioma.

2. Materials and methods

All solvents were either ACS-certified or HPLC grade solvents obtained from Sigma- Aldrich and used as received. MeO-DOTA was generously donated by Dr. Keith Frank (IsoTherapeutics Group Angleton, TX) and 1B4M-DTPA was kindly supplied by Dr. Martin Brechbiel (National Cancer Institute, Bethesda, MD). CHX-A"-DTPA and C- DOTA were purchased from Macrocyclics (Dallas, Texas), and used as received. [^{177}Lu]lutetium chloride was obtained from the University of Missouri-Columbia Research Reactor (Columbia, MO, USA) as a 0.05N HCl solution. Iodine-125 was purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA).

2.1 L8A4 mAb

EGFRvIII is a mutant form of the epidermal growth factor receptor that is characterized by the deletion of a segment of the external domain of wild-type EGFR consisting of amino acids 6 through 273 [20]. EGFRvIII is an attractive target for radioimmunotherapy because it is expressed on several types of human malignancies including glioma, head and neck cancer, breast and ovarian carcinoma, but not on normal tissues including the liver and others that express wild-type EGFR [21]. The development of the murine IgG1 anti-EGFRvIII mAb L8A4 have been described previously [22]. Briefly, mice were immunized with a synthetic peptide that mimicked the novel fusion junction characteristic of EGFRvIII followed several weeks later by inoculation with irradiated EGFRvIII-expressing transfected cells or cell membrane preparations. Purification, characterization, and documentation of the rapid internalization and intracellular degradation of L8A4 after binding to EGFRvIII- expressing cells, were carried out following previously described methods [23].

2.2 Conjugation of Chelates to mAb

Because of the propensity of these chelates to form inert complexes with endogenous metals, all buffers were prepared using procedures designed to minimize metal ion concentrations as described previously [18,24]. The L8A4 mAb-chelate conjugates were prepared by incubation of 0.5–2.5 mg L8A4 with a 10–20 fold molar excess of the bifunctional chelate in 10–12 mL 1H pH 8.6 conjugation buffer [18] for 24 h at room temperature. The conjugate were then subjected to centrifugal filtration using a Centricon concentrator (Amicon) with a 10 kDa cutoff to remove free chelate. During this process, the conjugation buffer was gradually replaced by 1H NH_4OAc (pH 7) buffer. The number of chelates per mAb was determined using a spectrophotometric assay based on the titration of a Pb(II)-Arsenazo(III) complex for MeO-DOTA and C-DOTA [25] and Y(III)-Arsenazo(III) complex for 1B4M-DTPA and CHX-A"-DTPA [26].

2.3 Radiolabeling of L8A4 with ^{177}Lu and ^{125}I

Lutetium-177 chloride (typically, 25 Ci/mg specific activity, 1.2 Ci/mL activity concentration) in 20 μL 0.05 M HCl, was diluted with 80 μL of 0.15 M NH_4OAc (pH 7) buffer, and an aliquot equal to ~ 2 $\mu\text{Ci}/\mu\text{g}$ mAb was transferred to an Eppendorff vial for radiolabeling. The mAb-chelate conjugate (200–300 μg) in 150 μL 0.15 M NH_4OAc (pH 7) buffer was added to the ^{177}Lu activity, buffered as described above, and incubated for 90 min at 25°C (room temperature). Incubation at higher temperatures and for longer periods did not significantly increase radiolabeling yields. The reaction was quenched with 3 μL of 0.15 M EDTA, and the labeled mAb was purified by passage through a Sephadex G25 PD-10 column (GE Health Care, Piscataway, NJ).

The L8A4 mAb was labeled with ^{125}I using the SGMIB method, a procedure that has been shown to increase retention of radioiodine in EGFRvIII-positive tumor cells after L8A4 internalization [19]. First, [^{125}I]SGMIB was prepared from the corresponding tin precursor and then about 1 mCi of the radioiodinated active ester was reacted with L8A4 (typically 80 μL , 1.3 mg/mL), previously dialyzed into 0.1 M borate buffer pH 8.5, for 20 min at room temperature [27]. Iodine-125-labeled L8A4 was isolated using a Sephadex G25 PD-10 column.

2.4 Determination of immunoreactive fraction

The immunoreactivity of each radiolabeled L8A4 preparation was determined by a magnetic bead assay using beads coated with the extracellular domain of EGFRvIII or as a measure of nonspecific binding, BSA [28]. Assays were done in paired-label format to directly compare the ^{125}I - and ^{177}Lu -labeled L8A4 preparations administered in each paired-label biodistribution experiment.

2.5 Biodistribution measurements

Animal studies were performed under a protocol approved by the Duke University Institutional Animal Care and Use Committee. BALB/c *nu/nu* mice were obtained from a closed breeding colony maintained at the Duke University Cancer Center Isolation Facility. Subcutaneous xenografts were established from the U87MG. EGFR human glioma cell line [29] by injection of 50 μL of tumor homogeneate in the flank through a 16 gauge needle. Based on flow cytometry experiments, disaggregated U87MG. EGFR xenografts express a mean of 2.5×10^5 EGFRvIII receptors per cell [30]. Biodistribution measurements were initiated when xenograft volumes were 200–400 mm^3 .

In the four paired-label tissue distribution studies, mice were injected with either ^{177}Lu -1B4M-DTPA-L8A4 (5 μCi , 1.8 $\mu\text{Ci}/\mu\text{g}$), ^{177}Lu -CHX-A"-DTPA-L8A4 (5 μCi , 2.5 $\mu\text{Ci}/\mu\text{g}$), ^{177}Lu -MeO-DOTA-L8A4 (5 μCi , 0.9 $\mu\text{Ci}/\mu\text{g}$), or ^{177}Lu -C-DOTA-L8A4 (6 μCi , 1.0 $\mu\text{Ci}/\mu\text{g}$) in tandem with [^{125}I]SGMIB-L8A4 (3–5 μCi , 0.7–3.2 $\mu\text{Ci}/\mu\text{g}$). Groups of five animals were killed by isoflurane overdose at 1, 2, 3, 4, 6 and 8 days post-injection. The 3-day time point was not performed in the ^{177}Lu -C-DOTA-L8A4 study. After dissection the tissues of interest were harvested, blot dried, and the samples were weighed and counted for both ^{177}Lu and ^{125}I activity using a dual-channel automated LKB 1282 gamma-counter (Turku, Finland) with decay and crossover correction capability. Tissue radioactivity levels were compared with injected dose (ID) standards, and the results were expressed as percentage of the injected dose per gram tissue (%ID/g). For each animal the ^{177}Lu to ^{125}I uptake ratio in tissues of interest also was determined. Differences in the radioactivity levels of two compounds administered simultaneously were analyzed for statistical significance using a paired *t*-test while an unpaired *t*-test was utilized to compare results from different experiments.

3. Results

3.1 Preparation of radiolabeled L8A4

Reaction of L8A4 with a 10–20:1 molar ratio of chelate to protein yielded an average number of chelates per mAb of 0.3 for CHX-A''-DTPA, 1.1 for 1B4M-DTPA 3.1 for C- DOTA and 2.7 for MeO-DOTA. Optimization of ^{177}Lu labeling of these conjugates was not performed in this study; nonetheless, labeling yields between 73–86% were obtained for the four chelates. Immunoreactive fractions determined by the magnetic bead assay also were similar, ranging from 60% for ^{177}Lu -CHX-A''-DTPA to 76% for ^{177}Lu -1B4M- DTPA. The immunoreactive fractions of the [^{125}I]SGMIB-L8A4 preparations used in the biodistribution experiments were in the same range, 60–71%. Prior to *in vivo* administration, all radiolabeled mAbs and mAb-chelate conjugates were analyzed by size-exclusion HPLC and demonstrated to elute as a single peak with a retention corresponding to that of unmodified L8A4 (data not shown).

3.2 Biodistribution of ^{177}Lu - and ^{125}I -labeled L8A4 in tumor bearing mice

A series of paired label tissue distribution experiments were performed in athymic mice bearing subcutaneous U87MG. EGFR human glioma xenografts. Our objective was to evaluate the potential utility of the two acyclic and two macrocyclic ligands for ^{177}Lu labeling of the anti-EGFRvIII L8A4 mAb, using co-administered [^{125}I]SGMIB-L8A4 as a common point of reference. The percent injected dose per gram (% ID/g) recovered from tumor and normal tissues 1, 4, and 8 days after injection of [^{125}I]SGMIB- L8A4 and L8A4 labeled with ^{177}Lu using 1B4M-DTPA, CHX-A''-DTPA, C-DOTA, and MeO-DOTA is summarized in Tables 1 through 4, respectively (Data for intermediate time points available upon request). With the exception of the study with C-DOTA, the % ID/g for the ^{177}Lu -labeled mAb in tumor was significantly higher ($p < 0.05$) than that for [^{125}I]SGMIB-L8A4 at all time points.

Because ^{177}Lu is a bone-seeking radiometal, it is important to minimize accumulation in this tissue in order to lessen radiotoxicity to proximal bone marrow. With regard to this criterion, we observed that the acyclic chelator CHX-A''-DTPA showed an increase in bone uptake over the course of the experiment starting from $5.95\% \pm 1.27\%$ ID/g on Day 1 to $7.67\% \pm 1.71\%$ ID/g on Day 8. Bone uptake values for mAb labeled via C- DOTA and 1B4M-DTPA were significantly lower ($p < 0.05$) than those observed with CHX-A''-DTPA. By far the lowest levels of ^{177}Lu bone-uptake were observed with MeO- DOTA, declining from $2.75\% \pm 0.40\%$ ID/g on Day 1 to $0.8\% \pm 0.3\%$ ID/g on Day 8. In other tissues, particularly the liver, spleen and kidneys, the four chelators showed a similar uptake pattern, declining by about a factor of two or three from Day 1 to Day 8.

Differences in the *in vivo* characteristics of the four ^{177}Lu -labeled L8A4-chelate conjugates also were evaluated by calculating their $^{177}\text{Lu}/^{125}\text{I}$ tissue uptake ratios. An advantage of using this parameter is that by normalizing each data set to the same co- administered [^{125}I]SGMIB-L8A4, it should be possible to minimize the effects of potential differences among groups of experimental animals such as tumor size. As shown in Figure 2, with the exception of the C-DOTA conjugate, $^{177}\text{Lu}/^{125}\text{I}$ uptake ratios in the blood did not vary considerably with time and were close to unity. In tumor, C-DOTA offered no significant delivery advantage compared with SGMIB; however, with the three other bifunctional chelates there was a significant tumor delivery advantage for ^{177}Lu labeling, particularly for 1B4M-DTPA and MeO-DOTA. For example, 6 days after injection, $^{177}\text{Lu}/^{125}\text{I}$ tumor uptake ratios were 5.1 ± 1.7 and 5.4 ± 1.6 for 1B4M-DTPA and MeO-DOTA, respectively. With the exception of CHX-A''-DTPA, $^{177}\text{Lu}/^{125}\text{I}$ uptake ratios in the bone were less than 2.0 from 1–4 days after injection; the lowest value observed at the end of the 8 day experiment was 3.2 ± 1.7 for MeO-DOTA. In general, $^{177}\text{Lu}/^{125}\text{I}$ uptake ratios in the kidney, liver and spleen increased with time, reflecting significantly higher retention of ^{177}Lu compared with ^{125}I in these organs. Except

for the results for C-DOTA in the kidney, $^{177}\text{Lu}/^{125}\text{I}$ uptake ratios in these tissues reached values greater than 4 by 6 days after injection.

In order to assess the selectivity of tumor targeting for the ^{177}Lu -labeled L8A4- chelate conjugates as well as the co-administered [^{125}I]SGMIB-L8A4 preparations, tumor:normal tissue ratios for the four paired-label experiments were calculated. The results for the blood, brain, bone, spleen, liver and kidneys at 1, 4 and 8 days are summarized in Figure 3. In spleen, liver and kidney, higher Tumor:normal tissue ratios generally were observed with the radioiodinated mAb while the opposite behavior was seen in the blood and the brain. In most tissues and at most time points, use of the MeO-DOTA chelate for labeling L8A4 with ^{177}Lu resulted in the most selective targeting. Table 5 summarizes tumor:normal tissue ratio results at 3 days after injection for ^{177}Lu - MeO-DOTA-L8A4 and co-administered [^{125}I]SGMIB-L8A4 in a more comprehensive set of tissues. Tumor:normal tissue ratios for [^{125}I]SGMIB-L8A4 were marginally higher in liver, spleen and kidneys, however, two- to threefold more selective targeting was seen in all other tissues with ^{177}Lu -MeO-DOTA-L8A4.

4. Discussion

The vast majority of targeted radiotherapeutic studies have involved β -emitting radionuclides with ^{131}I and ^{90}Y being the most extensively investigated β -emitters based at least in part on their widespread availability. Now that ^{177}Lu is also routinely available at a reasonable cost, it has emerged as an attractive alternative to ^{90}Y and ^{131}I for targeted radiotherapy. Moreover, recent studies have demonstrated practical advantages for ^{177}Lu for labeling peptides [31] as well as improved therapeutic efficacy with ^{177}Lu as a label for MOv18 mAb compared with ^{131}I and ^{90}Y [32]. The later observation is consistent with the favorable aspects of the ^{177}Lu emissions spectrum – low β -particle energy combined with low γ -ray energies and intensities - which should facilitate maximizing energy deposition in tumor while minimizing toxicity to neighboring normal tissues.

A setting in which ^{177}Lu emission characteristics should be of particular benefit is the treatment of minimum residual disease, where efficient delivery of curative doses of radiation to small tumor foci is of paramount importance. The irradiation of residual glioma cells remaining after the resection of brain tumors is a notable example that has been evaluated in several clinical studies, with the majority of these involving anti- tenascin C mAbs labeled with ^{131}I [10]. The long-term objective of the current study is to develop an improved targeted radiotherapeutic for brain tumor treatment by not only replacing ^{131}I with ^{177}Lu but also utilizing a molecular target with greater tumor specificity. Unlike tenascin-C, which is expressed on liver and spleen, EGFRvIII is not expressed on normal tissues [21], making mAbs such as L8A4 that recognize this mutant receptor attractive candidates for radioimmunotherapy.

A variety of acyclic and macrocyclic bifunctional chelates have been utilized for labeling mAbs with trivalent radiometals; our goal was to determine whether the nature of the ligand had an impact on its suitability for labeling L8A4 mAb with ^{177}Lu . Most investigations regarding bifunctional chelators and radiometals have dealt with ^{90}Y and in general, macrocyclic chelators show greater kinetic inertness than acyclic chelators [12]. The *in vivo* properties of mAbs and mAb fragments labeled with ^{177}Lu using a variety of chelators also has been evaluated. For example, Milenic et al [16] compared PA-DOTA and C-DOTA with the acyclic chelator CHX-A"-DTPA conjugated for labeling the HuCC49 CH2 fragment with ^{177}Lu . The ^{177}Lu -C-DOTA and ^{177}Lu -CHX-A"-DTPA conjugates exhibited good targeting whereas the ^{177}Lu -PA-DOTA conjugate was not stable *in vivo*. In a similar type of comparison utilizing NHS-DOTA, MeO-DOTA and 1B4M-DTPA for labeling both the murine and chimeric forms of anti-tenascin mAb 81C6 with ^{177}Lu , we observed that the use of MeO-DOTA was shown to provide the best tumor:normal tissue ratios with both constructs [17].

Unlike the mAbs and MAb fragment evaluated in these chelate comparison studies, the L8A4 mAb is rapidly internalized into EGFRvIII-expressing tumor cells where it undergoes extensive intracellular catabolism [23]. Thus, in addition to ^{177}Lu complex stability and normal tissue clearance of labeled catabolites, maximizing the intracellular trapping of ^{177}Lu after internalization of the mAb-EGFRvIII complex will be an important factor influencing the selection of the best bifunctional chelate for labeling L8A4 with ^{177}Lu . Interpretation of the effect of the last variable should be facilitated by comparison to the results from a previous study [18] in which we evaluated the intracellular trapping of radioactivity by U87. EGFR glioma cells after incubation with ^{177}Lu - and ^{125}I -labeled L8A4 prepared via a variety of methods including all those utilized herein.

Because both intracellular trapping *in vitro* and tumor uptake *in vivo* are time dependent, and different observation periods were used for the cell culture (1–24 h) and tissue distribution (1–8 Days) experiments, only general trends might be discernable even though the same U87. EGFR glioma cell model was utilized in both cases. In the internalization assays, $^{177}\text{Lu}/^{125}\text{I}$ intracellular activity ratios reflected higher uptake for [^{125}I]SGMIB-L8A4 compared with mAb labeled via 1B4M-DTPA, CHX-A"-DTPA and C- DOTA from 1–8 h with the opposite behavior seen at 16 and 24 h [18]. In contrast, $^{177}\text{Lu}/^{125}\text{I}$ intracellular activity ratios were greater than 1 at all points. After a 24-h incubation, $^{177}\text{Lu}/^{125}\text{I}$ ratios were: C-DOTA, 1.76 ± 0.24 ; MeO-DOTA, 2.09 ± 0.17 ; 1B4M-DTPA, 2.34 ± 0.39 ; CHX-A"-DTPA, 2.86 ± 0.26 . The fact that in the current *in vivo* study, the $^{177}\text{Lu}/^{125}\text{I}$ uptake ratios in U87. EGFR xenografts (Figure 2) were the lowest for the four bifunctional chelates at all time points is consistent with these results. However, the fact that the $^{177}\text{Lu}/^{125}\text{I}$ tumor uptake ratios for MeO-DOTA and 1B4M-DTPA are higher than those for CHX-A"-DTPA would not have been predicted based on the *in vitro* internalization assay results at 24 h. On the other hand, the rank order of the $^{177}\text{Lu}/^{125}\text{I}$ tumor uptake ratios are in good agreement with the $^{177}\text{Lu}/^{125}\text{I}$ intracellular activity ratios observed at early time points, at which time MeO-DOTA and 1B4M-DTPA have higher values than CHX-A"-DTPA. Thus, although internalization assays may be of value for predicting the relative merit of labeling methods for internalizing mAbs; this must be done with caution.

Although our intended clinical application involves direct injection of labeled mAbs into surgically created tumor resection cavities, leakage of radioactivity from the cavity does occur with time [10], making it important to select a labeling method that maximizes tumor:normal tissue ratios. In our previous studies with ^{177}Lu -labeled anti- tenascin mAb 81C6, which reacts with a non-internalizing molecular target, the selectivity of tumor uptake was dependent both on the properties of the chelate and the form of the mAb. With the more stable chimeric construct, both 1B4M-DTPA and MeO- DOTA were effective in providing acceptable levels of ^{177}Lu in bone, liver, spleen and kidneys; however, with murine 81C6, labeling via MeO-DOTA resulted in significantly lower ^{177}Lu levels in these tissues. The results of the current study with the rapidly internalizing L8A4 mAb also demonstrate chelate-dependent differences in normal organ uptake expressed in terms of % ID/g, $^{177}\text{Lu}/^{125}\text{I}$ uptake ratio and tumor:normal tissue ratio.

The magnitude of tumor uptake and tumor:normal tissue ratios probably are the most relevant parameters for evaluating the relative merit of these radiolabeled L8A4 conjugates for targeted radiotherapy. Although tumor:tissue ratios for [^{125}I]SGMIB-L8A4 in spleen, liver and kidneys are more favorable than those observed with any of the ^{177}Lu -labeled mAb conjugates, this must be balanced by the fact that for 1B4M-DTPA and MeO-DOTA, the magnitude of tumor uptake is up to 4 to 5 times higher than seen with the radioiodinated mAb. Given that the anticipated non-intravenous route of delivery will delay exposure to normal tissue and that ^{177}Lu has better emission properties than ^{131}I , the potential benefits of labeling L8A4 mAb with ^{177}Lu via these two chelates appear to outweigh the disadvantages. Furthermore,

tumor:normal tissue ratios for ^{177}Lu -1B4M-DTPA-L8A4 and to an even greater extent, ^{177}Lu -MeO-DOTA-L8A4, were higher than those for [^{125}I]SGMIB-L8A4 in most other tissues. Finally, uptake and retention of ^{177}Lu in blood and bone was lower for ^{177}Lu -MeO-DOTA-L8A4 than ^{177}Lu -1B4M-DTPA-L8A4, which should help decrease bone marrow toxicity. Based on these observations, we have selected MeO-DOTA as the bifunctional chelate for labeling L8A4 mAb with ^{177}Lu for our planned clinical radioimmunotherapy trial in patients with malignant brain tumors.

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Figure 1.
Structures of the bifunctional chelators CHX-A''-DTPA, 1B4M-DTPA, C-DOTA and MeO-DOTA used in this study.

Figure 2. Ratio of ^{177}Lu to ^{125}I activity in U87. EGFR glioma xenografts and selected normal tissues following four paired label experiments. [^{125}I]SGMIB-L8A4 co-administered with L8A4 mAb labeled with ^{177}Lu using either 1B4M-DTPA, CHX-A''-DTPA, C-DOTA, or MeO-DOTA.

Figure 3. Tumor:normal tissue uptake ratios in athymic mice with subcutaneous U87. EGFR glioma xenografts from four paired label experiments. [^{125}I]SGMIB-L8A4 co-administered with L8A4 mAb labeled with ^{177}Lu using either 1B4M-DTPA, CHX-A''-DTPA, C-DOTA, or MeO-DOTA.

Table 1

Paired-label biodistribution of L8A4 labeled with ^{177}Lu using 1B4M-DTPA and with ^{125}I using SGMIB in athymic mice with subcutaneous U87MG. EGFR human glioma xenografts

Nuclide	Tissue	Day 1	Day 4	Day 8
^{177}Lu	Blood	19.78 ± 2.94	10.75 ± 2.88	1.35 ± 0.94
	Liver	8.36 ± 0.74	7.52 ± 2.18	4.16 ± 0.54
	Spleen	5.64 ± 1.13	4.62 ± 1.73	2.01 ± 1.01
	Kidneys	8.06 ± 1.03	5.34 ± 1.25	2.61 ± 0.32
	Lung	6.87 ± 1.61	6.00 ± 3.12	0.93 ± 0.45
	Bone	3.58 ± 1.44	2.44 ± 0.71	1.82 ± 0.52
	Muscle	1.64 ± 0.08	0.88 ± 0.22	0.30 ± 0.07
	Brain	0.58 ± 0.13	0.40 ± 0.12	0.07 ± 0.05
	Thyroid ^a	0.50 ± 0.13	0.27 ± 0.07	0.11 ± 0.04
	Tumor	31.58 ± 7.24	29.24 ± 6.03	15.27 ± 5.16
^{125}I	Blood	18.78 ± 2.24	10.64 ± 2.86	1.29 ± 0.87
	Liver	5.11 ± 0.86	2.89 ± 0.75	0.42 ± 0.32
	Spleen	4.48 ± 0.72	2.26 ± 0.80	0.28 ± 0.19
	Kidneys	4.73 ± 0.68	2.74 ± 0.69	0.39 ± 0.29
	Lung	6.81 ± 1.40	4.28 ± 0.78	0.58 ± 0.41
	Bone	3.02 ± 0.99	1.53 ± 0.49	0.33 ± 0.18
	Muscle	1.66 ± 0.08	0.85 ± 0.19	0.15 ± 0.10
	Brain	0.55 ± 0.13	0.40 ± 0.10	0.05 ± 0.04
	Thyroid ^a	0.37 ± 0.30	0.31 ± 0.07	0.02 ± 0.03
	Tumor	21.64 ± 4.53	9.60 ± 1.72	2.86 ± 0.98

Values presented as percent injected dose per gram tissue (% ID/g), mean " standard deviation; five mice per group.

^aPercent injected dose in the thyroid.

Table 2

Paired-label biodistribution of L8A4 labeled with ^{177}Lu using CHX-A"-DTPA and with ^{125}I using SGMIB in athymic mice with subcutaneous U87MG. EGFR human glioma xenografts

Nuclide	Tissue	Day 1	Day 4	Day 8
^{177}Lu	Blood	15.18 ± 3.30	6.10 ± 2.47	0.99 ± 0.71
	Liver	8.53 ± 2.39	9.36 ± 3.59	4.45 ± 0.87
	Spleen	5.98 ± 1.86	3.97 ± 0.53	1.84 ± 0.70
	Kidneys	6.07 ± 1.56	3.96 ± 1.24	2.00 ± 0.37
	Lung	5.39 ± 1.28	2.44 ± 0.94	0.56 ± 0.23
	Bone	5.95 ± 1.27	6.55 ± 1.77	7.56 ± 1.88
	Muscle	1.30 ± 0.44	0.64 ± 0.21	0.19 ± 0.05
	Brain	0.46 ± 0.12	0.21 ± 0.08	0.05 ± 0.03
	Thyroid ^a	0.44 ± 0.13	0.24 ± 0.07	0.14 ± 0.07
	Tumor	36.28 ± 6.60	29.00 ± 7.07	8.67 ± 3.42
^{125}I	Blood	16.24 ± 3.73	8.18 ± 3.25	1.50 ± 1.32
	Liver	4.43 ± 0.77	2.14 ± 0.86	0.46 ± 0.37
	Spleen	3.77 ± 1.43	1.51 ± 0.52	0.35 ± 0.30
	Kidneys	3.87 ± 1.03	2.16 ± 1.18	0.42 ± 0.34
	Lung	5.70 ± 1.64	2.90 ± 1.30	0.50 ± 0.40
	Bone	2.51 ± 0.86	1.24 ± 0.51	0.36 ± 0.27
	Muscle	1.37 ± 0.58	1.29 ± 0.49	0.16 ± 0.13
	Brain	0.50 ± 0.13	0.28 ± 0.10	0.09 ± 0.09
	Thyroid ^a	0.50 ± 0.13	0.27 ± 0.09	0.09 ± 0.02
	Tumor	27.56 ± 4.82	13.09 ± 2.97	3.39 ± 2.19

Values presented as percent injected dose per gram tissue (% ID/g), mean " standard deviation; five mice per group.

^aPercent injected dose in the thyroid.

Table 3

Paired-label biodistribution of L8A4 labeled with ^{177}Lu using C-DOTA and with ^{125}I using SGMIB in athymic mice with subcutaneous U87MG. EGFR human glioma xenografts

Nuclide	Tissue	Day 1	Day 4	Day 8
^{177}Lu	Blood	10.95 ± 1.53	2.47 ± 1.80	1.33 ± 1.60
	Liver	13.00 ± 4.79	9.34 ± 1.71	5.39 ± 2.54
	Spleen	5.43 ± 1.50	3.61 ± 1.02	2.47 ± 1.84
	Kidneys	4.26 ± 0.70	1.28 ± 0.60	1.22 ± 0.67
	Lung	4.21 ± 1.28	0.99 ± 0.70	0.72 ± 0.67
	Bone	4.49 ± 0.84	1.21 ± 0.53	2.39 ± 1.55
	Muscle	1.32 ± 0.26	0.30 ± 0.14	0.25 ± 0.16
	Brain	0.37 ± 0.05	0.22 ± 0.37	0.05 ± 0.04
	Thyroid ^a	0.17 ± 0.09	0.04 ± 0.03	0.06 ± 0.05
	Tumor	23.52 ± 3.14	12.04 ± 6.29	7.47 ± 6.89
^{125}I	Blood	13.93 ± 3.17	8.16 ± 1.08	3.82 ± 3.70
	Liver	4.15 ± 0.85	1.78 ± 0.25	1.14 ± 1.07
	Spleen	2.89 ± 0.59	1.62 ± 0.18	0.81 ± 0.78
	Kidneys	3.47 ± 0.63	1.61 ± 0.36	1.00 ± 0.93
	Lung	5.46 ± 1.86	2.49 ± 0.53	1.60 ± 1.50
	Bone	2.47 ± 0.21	0.87 ± 0.17	0.57 ± 0.41
	Muscle	1.64 ± 0.32	0.59 ± 0.09	0.40 ± 0.32
	Brain	0.48 ± 0.08	0.41 ± 0.46	0.13 ± 0.10
	Thyroid ^a	0.29 ± 0.11	0.22 ± 0.10	0.18 ± 0.12
	Tumor	20.76 ± 5.02	11.24 ± 3.09	7.37 ± 6.27

Values presented as percent injected dose per gram tissue (% ID/g), mean " standard deviation; five mice per group.

^aPercent injected dose in the thyroid.

Table 4

Paired-label biodistribution of L8A4 labeled with ^{177}Lu using MeO-DOTA and with ^{125}I using SGMIB in athymic mice with subcutaneous U87MG. EGFR human glioma xenografts

Nuclide	Tissue	Day 1	Day 4	Day 8
^{177}Lu	Blood	15.35 ± 2.41	3.73 ± 2.88	0.70 ± 0.62
	Liver	7.88 ± 1.56	4.67 ± 1.55	2.89 ± 0.71
	Spleen	4.53 ± 0.52	2.72 ± 1.00	1.44 ± 0.55
	Kidneys	7.04 ± 0.69	3.53 ± 0.88	2.34 ± 0.32
	Lung	5.01 ± 1.82	1.34 ± 0.73	0.43 ± 0.27
	Bone	2.75 ± 0.40	0.90 ± 0.39	0.82 ± 0.29
	Muscle	1.65 ± 0.35	0.46 ± 0.19	0.15 ± 0.08
	Brain	0.51 ± 0.12	0.11 ± 0.07	0.04 ± 0.03
	Thyroid ^a	0.26 ± 0.13	0.06 ± 0.05	0.02 ± 0.01
	Tumor	38.77 ± 8.55	26.30 ± 9.68	10.37 ± 4.05
^{125}I	Blood	15.62 ± 2.32	4.09 ± 3.22	0.81 ± 0.71
	Liver	4.07 ± 0.50	0.98 ± 0.71	0.31 ± 0.26
	Spleen	2.74 ± 0.49	0.76 ± 0.54	0.22 ± 0.17
	Kidneys	3.74 ± 0.57	0.88 ± 0.55	0.25 ± 0.20
	Lung	5.16 ± 1.83	1.23 ± 0.80	0.34 ± 0.29
	Bone	2.61 ± 0.38	0.63 ± 0.38	0.26 ± 0.16
	Muscle	1.67 ± 0.40	0.41 ± 0.20	0.11 ± 0.08
	Brain	0.52 ± 0.11	0.11 ± 0.07	0.03 ± 0.03
	Thyroid ^a	0.31 ± 0.13	0.12 ± 0.06	0.08 ± 0.02
	Tumor	25.19 ± 4.77	7.52 ± 3.66	2.22 ± 1.44

Values presented as percent injected dose per gram tissue (% ID/g), mean " standard deviation; five mice per group.

^aPercent injected dose in the thyroid.

Table 5

Tumor-to-normal tissue ratios of radioactivity in athymic mice bearing subcutaneous U87. EGFR human glioma xenografts 3 days after injection of L8A4 mAb labeled with ^{177}Lu using MeO-DOTA and ^{125}I using SGMIB

Tissue	Lu MeO-DOTA	I SGMIB
Liver	7.8 " 4.1	10.4 " 2.6
Spleen	14.6 " 4.6	14.9 " 3.9
Lungs	25.4 " 3.8	7.6 " 2.5
Heart	39.4 " 8.6	10.8 " 2.2
Kidney	9.4 " 4.1	11.0 " 2.4
Stomach	214 " 47	72.1 " 17.1
Small Intestine	84.1 " 21.6	33.0 " 8.5
Large Intestine	96.8 " 37.7	37.7 " 13.7
Blood	10.8 " 3.3	2.9 " 0.7
Muscle	69.6 " 22.0	21.4 " 6.9
Bone	35.9 " 9.7	15.4 " 4.0
Brain	350 " 50	88.8 " 17.4