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Effect of Chelator Conjugation Level and Injection Dose on Tumor and Organ Uptake of ¹¹¹In Labeled MORAb-009, an Anti-mesothelin Antibody

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Abstract

Introduction—Radiolabeling of a monoclonal antibody (mAb) with a metallic radionuclide requires the conjugation of a bifunctional chelator to the mAb. The conjugation, however, can alter the physical and immunological properties of the mAb, consequently affecting its tumor targeting pharmacokinetics. In this study, we investigated the effect of the amount of 2-(*p*-isothiocyanatobenzyl)-cyclohexyl-diethylenetriamine-pentaacetic acid (CHX-A'') conjugated to MORAb-009, a mAb directed against mesothelin and the effect of MORAb dose on the biodistribution of ¹¹¹In labeled MORAb-009.

Methods—We used nude mice bearing A431/K5 tumor as a mesothelin-positive tumor model and A431 tumor as a mesothelin-negative control. To find the optimal level of CHX-A'' conjugation, CHX-A''-MORAb-009 conjugates with 2.4, 3.5, and 5.5 CHX-A'' molecules were investigated. To investigate the effect of injected MORAb-009 dose on neutralizing the shed-mesothelin in the circulation, the biodistribution studies were performed after the *i.v.* co-injection of the ¹¹¹In labeled MORAb-009 (2.4 CHX-A''/MORAb-009) with three different doses, 0.2, 2, and 30 µg of MORAb-009.

Results—The tumor uptake in A431/K5 tumor was 4 times higher than that in A431 tumor, indicating that the tumor uptake in A431/K5 was mesothelin-mediated. The conjugate with 5.5 CHX-A'' showed a lower isoelectric point (pI) and lower immunoreactivity (IR) than the 2.4 CHX-A'' conjugate. These differences were reflected in biodistribution of the ¹¹¹In label. The ¹¹¹In labeled MORAb-009 conjugated with 2.4 CHX-A'' produced higher tumor uptake, and lower liver and spleen uptakes than the 5.5 CHX-A'' conjugate. The biodistribution studies also revealed that the tumor uptake was significantly affected by the injected MORAb-009 dose and tumor size. The 30 µg dose produced higher tumor uptake than the 0.2 and 2 µg doses whereas the 30 µg dose produced lower liver and spleen uptakes than the 0.2 µg dose.

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Conclusion—This study demonstrates that the number of chelate conjugation and the injected dose are two important parameters to achieve high tumor and low non-target organ uptake of ^{111}In labeled MORAb-009. This study also suggests that the injected dose of mAb could be individualized based on the tumor size or the blood level of shed-antigen in a patient to achieve the ideal tumor-to-organ radioactivity ratios.

Keywords

^{111}In -anti-mesothelin antibody (^{111}In -MORAb-009); chelator conjugation; biodistribution; dose effect

1. Introduction

Monoclonal antibodies (mAbs) labeled with radionuclides have been actively investigated for diagnosis and therapy [1-8]. In order to radiolabel a mAb with a metallic radionuclide, a bifunctional chelator with a high metal-chelate stability constant is conjugated to the mAb to ensure that *in vivo* distribution of the radiolabel closely reflects that of the mAb [9-15]. From the standpoint of radiolabeling, it is preferable to conjugate a higher amount of chelator per mAb in order to improve a radiolabeling efficiency. However, the conjugation of more chelator/mAb can alter the physical, biological, and pharmacokinetic properties of the mAb. For instance, the conjugation of a polyaminocarboxylic acid, DTPA, which is a common bifunctional chelator used for ^{111}In labeling, could increase net negative charges in the mAb, thereby decreasing the isoelectric point (pI) of the mAb [14]. The conjugation of the chelator also increases the probability of decreasing the binding affinity of the mAb to its antigen [16-18]. Finally, a high number of chelator/mAb could change the *in vivo* clearance pharmacokinetics due to the uptake of the conjugate by the reticulo-endothelial system in liver and spleen [19].

In this study, we conjugated MORAb-009, a chimeric mAb directed against mesothelin with a bifunctional chelator, CHX-A'', for radiolabeling with ^{111}In . Mesothelin, a glycosyl-phosphatidyl inositol (GPI)-linked membrane protein of 40 kDa, is a tumor differentiation antigen whose expression in normal human tissues is limited to the mesothelial cells lining the pleura, pericardium and peritoneum [20, 21]. Mesothelin is over-expressed in almost all pancreatic adenocarcinomas and mesotheliomas, in >70% of ovarian adenocarcinomas, in many non-small cell lung cancers and some colorectal cancers [20, 22]. MORAb-009 is a high affinity chimeric (mouse/human) antibody which shares 82.6% amino acid sequence identity to a human IgG1 κ and is currently in clinical trials for treatment of patients with mesothelin expressing cancer [23]. MORAb-009 was synthesized by grafting the heavy and light chain variable regions of SS1(scFv) of mouse anti-mesothelin antibody, which was derived from a mouse immune library and further improved by *in vitro* maturation, with human IgG1 and kappa constant regions [22].

We are interested in the use of ^{111}In - and ^{90}Y -labeled MORAb-009 for the radioimmuno-detection and -therapy of mesothelin-expressing tumors. Since the level of CHX-A'' conjugation could affect the radiolabeling efficiency, isoelectric point, and immunoreactivity of MORAb-009, which could affect tumor targeting pharmacokinetics, we studied the optimal level of CHX-A'' conjugation on MORAb-009. Imaging could be complicated by the shedding of mesothelin into the blood of patients [24] which has also been demonstrated in mouse models of mesothelin-expressing tumor xenografts [25]. Therefore, we also investigated the dose of MORAb-009 needed to neutralize shed circulating mesothelin, thereby producing higher tumor-to-non-tumor ratios of ^{111}In labeled MORAb-009. Here, we report *in vitro* and *in vivo* results obtained by optimizing the level of CHX-A'' conjugation

and the injection dose of unconjugated MORAb-009 to achieve a high tumor uptake in a nude mouse model of mesothelin-expressing A431/K5 tumors.

2. Materials and Methods

2.1. Conjugation of CHX-A'' to MORAb-009

MORAb-009 was obtained from Morphotek, Inc. (Exton, PA). MORAb-009 (0.022 mM) was reacted with CHX-A'' (Macrocyclics, Dallas, TX) at a 50-times molar excess in 0.1 M sodium bicarbonate (1.5 ml), pH 9.5 at 25° C for 0.5 h, 1.5 h and 3 h. Each CHX-A''-MORAb-009 conjugate was purified with a size exclusion Zeba Desalt Spin column (Pierce, Rockford, IL) or a microcon filter with a 30 kDa cutoff (Millipore, Bedford, MA). The column or the filter was pretreated with 25 mg BSA containing 1 µmol DTPA to block nonspecific protein binding sites and remove potential metal contaminants, and then washed with metal free sodium acetate of 0.1 M (pH 4.5). The mAb and CHX-A'' concentrations were measured according to the method of Bradford, et al. [26] and the method of Pippin, et al. [27], respectively to assess the level of CHX-A'' conjugated per MORAb-009.

2.2. Radiolabeling

Purified CHX-A''-MORAb-009 (1 mg/ml, 6.7 µM) was labeled with $^{111}\text{InCl}_3$ in 0.2 M sodium acetate (pH 4.5) at 25 °C for 1 h. DTPA at a final concentration of 0.2 mM was then added to the reaction solution to bind possible free ^{111}In ion [12]. The labeled product was purified with a size exclusion PD-10 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) eluted with PBS. The radiolabeling yield and the radiochemical purity were assessed by size exclusion HPLC (Gilson, Middleton, WI) equipped with a size exclusion TSK gel G3000SWXL column (7.8 × 300 mm, 5 µm, TOSOH Bioscience, Japan; 0.067 M sodium phosphate/0.15 M sodium chloride, pH 6.8; 1.0 ml/min), a UV monitor, and an on-line flow radioactivity detector (Bioscan Inc., Washington, DC) before and after the purification. The radiolabeling yield (> 90%) was determined based on the distribution of ^{111}In between CHX-A''-MORAb-009 (retention time, 9.0 min) and DTPA (retention time, 13.0 min) on the HPLC profiles. The radiochemical purity of the purified product was > 98% and the specific activity was 5~10 µCi/µg of MORAb-009.

2.3. SDS-PAGE Analysis

To estimate the possible change in the MORAb-009 structure caused by the CHX-A'' conjugation, the electrophoresis was performed with XCell *SureLock*® Mini-Cell (Invitrogen CA, USA). Aliquot (10 µg/10 µL) of the MORAb-CHX-A'' conjugates from the 0.5-, 1.5-, and 3-h reactions were treated with or without 5% 2-mercaptoethanol (ICN Biomedicals Inc., Aurora, OH) at 90 °C for 5 min to reduce the interchain disulfide bonds. The samples and molecular weight standards were loaded on a 4-10% Tris-glycine gel (Invitrogen, Carlsbad, CA) in a running buffer containing 25 mM Tris, 192 mM glycine, pH 8.3, and 0.1% (w/v) SDS (Bio-Rad, Hercules, CA). The loaded gels were run at 125 V for 2 h. The gels were stained for proteins with colloidal Coomassie blue (Invitrogen, CA, USA). The mobility of the conjugated and unconjugated MORAb-009 were compared with molecular weight standards (SeeBluePlus2, Invitrogen, Carlsbad, CA); the standards include myosin, phosphorylase B, BSA, glutamic dehydrogenase, alcohol dehydrogenase, carbonic anhydrase, myoglobin lysozyme, aprotinin, and insulin B chain).

2.4. Isoelectric Focusing

The isoelectric focusing was performed with XCell *SureLock*® Mini-Cell (Invitrogen CA, USA) following the manufacture's instruction. Aliquots (10 µg/ 10 µL) of the CHX-A''-MORAb-009 conjugates from the 0.5-, 1.5- and 3-h reactions, unconjugated MORAb, and standard markers with pI values ranging from 3.5 to 9.3 were loaded on a gel with a pI

ranging from 3 to 10 (Novex®, Invitrogen, CA, USA). The loaded gels were run at 100 V for 1 h, 200 V for 1 h and then 500 V for 30 min. The gels were stained with Colloidal Blue (Novex®, Invitrogen, Carlsbad, CA), and the pI values of the products were determined by comparing their bands to the standard bands.

2.5. Immunoreactivity Determination

The immunoreactivity of ^{111}In -CHX-A''-MORAb-009 with different levels of CHX-A'' conjugation was determined by a modified cell-binding assay of Lindmo, et al. [28]. Aliquots (5 g/50 μl) of the conjugate samples were incubated side-by-side with an increasing number of A431/K5 cells (2×10^4 - 1×10^6) in 100 μl of PBS with 1% BSA at 4°C for 3 hours. Nonspecific binding to the cells was determined by performing the cell-binding assays under a condition of antibody excess (25 μg of MORAb-009). The maximum specific cell-bound counts, corrected for the non-specific binding and expressed as a percentage of the total counts, were taken as the immunoreactivity.

2.6. Tumor Model

A431/K5 lines were obtained from Dr. Ira Pastan, NCI. A431/K5 cells were derived from the A431 cell line by co-transfection of human epidermoid cancer A431 cells (American Type Culture Collection) with a plasmid encoding mesothelin and a neomycin-resistance gene (data not shown). A431/K5 and A431 cells were grown as previously described [29]. Briefly, A431/K5 and A431 cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, penicillin (100 IU/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) and G-418 (Geneticin, 750 $\mu\text{g}/\text{ml}$). Both cell lines were grown at 37°C in a moist atmosphere with 5% CO_2 . Tumor xenografts were established by inoculation of 2×10^6 A431/K5 and A431 cells in 0.1 ml PBS subcutaneously into the right hind flank and the left hind flank of athymic mice (NCI-DCT, Frederick, MD), respectively.

2.7. Biodistribution Studies

Animal experiments were performed under a protocol approved by the NIH Animal Care and Use Committee. For comparative biodistribution studies with ^{111}In -labeled MORAb-009 conjugate with 2.4 CHX-A'' molecules and that with 5.5 CHX-A'' molecules, groups ($n = 5$ -14 mice/group) of mice with A431/K5 tumors were injected *iv* with each of ^{111}In -labeled MORAb-009 conjugates (2 $\mu\text{Ci}/0.2 \mu\text{g}$) mixed with unlabeled MORAb-009 (30 μg total) in 0.2 mL PBS containing 1% BSA when the tumor size reached approximately 200 mg. This high amount (30 μg) of unlabeled MORAb-009 was co-injected to block shed-mesothelin in blood. In separate experiments, biodistribution studies were performed to investigate the effect of co-injected cold MORAb-009 dose on the tumor uptake of ^{111}In -labeled MORAb-009 when the tumor reached up to 400 mg. Groups ($n = 5$ -14 mice/group) of mice with A431/K5 tumor were injected *iv* with the ^{111}In -labeled MORAb-009 with 2.4 CHX-A'' molecules (2 $\mu\text{Ci}/0.2 \mu\text{g}$) mixed with unlabeled MORAb-009 dose to get the total MORAb-009 dose ranging from 0.2, 2 to 30 μg in 0.2 mL PBS containing 1% BSA. To demonstrate that the tumor uptake of ^{111}In -CHX-A''-MORAb-009 in A431/K5 tumor is specific uptake mediated by binding to mesothelin, a group of nude mice implanted with the mesothelin-positive A431/K5 tumor in the right hind flank and the mesothelin-negative A431 tumor in the left hind flank were injected with ^{111}In -CHX-A''-MORAb-009 (2 $\mu\text{Ci}/0.2 \mu\text{g}$). The animals were euthanized at designated time points by CO_2 inhalation and exsanguinated by cardiac puncture before dissection. Blood and various organs were removed and weighed, and their decay corrected radioactivity counts were measured with a gamma-counter (Wallac, Inc., Perkin-Elmer, Inc., Boston, MA). The percentage of injected dose per gram (% ID/g) of the blood or each organ was calculated and normalized to a 20-gram mouse. The whole-body (WB) radioactivity count was obtained by adding the radioactivity of all organs and that of the carcass measured

by the gamma-counter, and the whole-body radioactivity was expressed as the percentage of the injected dose (% ID).

2.8. Statistical Analysis

Statistical analysis was performed using ANOVA for comparing multiple groups, and the Student's *t* test was performed for unpaired data between two groups. All tests were two-sided, and a probability value (*P*) of less than 0.05 was considered significant.

3. Results

3.1. Chemistry and Product Analysis

MORAb-009 was conjugated with CHX-A'' at lysine residues using the isothiocyanate conjugation chemistry. The conjugation reaction was performed at pH 9.5 to avoid a potential solubility problem of MORAb-009 if the reaction was run at a pH near the isoelectric point (pI 8.3) of MORAb-009. The conjugation reaction was run at a molar excess of CHX-A'' to shorten the conjugation reaction time and to avoid the exposure of MORAb-009 to higher pH for a prolonged time. The reaction was stopped at 3 different time points, 0.5, 1.5, and 3 h. The conjugates were then purified and the level of CHX-A'' conjugation was calculated by determining the CHX-A'' concentration with the Arsenazo method and the MORAb concentration with the Bradford method. The conjugation reaction produced 2.4, 3.5, and 5.5 CHX-A'' molecules per MORAb at 0.5, 1.5 and 3 h, respectively. SDS-PAGE of the conjugates with and without 2-mercaptoethanol treatment indicated that these CHX-A'' conjugates have electrophoretic mobilities similar to that of unmodified MORAb-009 (Figure 1). However, isoelectric focusing experiments showed that the pI of MORAb-009 decreased proportionally to the level of CHX-A'' conjugation; the major pI was centered at ~8.3, ~8.3~7.6, ~7.3, and ~6.3 for MORAb-009 without CHX-A'', a conjugate with 2.4 CHX-A''/MORAb-009, 3.5 CHX-A''/MORAb-009, and 5.5 CHX-A''/MORAb-009, respectively (Fig. 2). Among these conjugates, we compared two conjugates, 2.4 CHX-A'' molecules/MORAb-009 and 5.5 CHX-A'' molecules/MORAb-009 for radiolabeling efficiency, immunoreactivity and pharmacokinetics. The labeling yield was equally high, >94%, for both the 2.4 CHX-A'' and 5.5 CHX-A'' conjugates (Table 1). However, the immunoreactivity of the 2.4 CHX-A'' ($88.3 \pm 4.5\%$) was significantly higher than that of the 5.5 CHX-A'' conjugate ($81.1 \pm 0.9\%$) ($P < 0.05$) (Table 1).

3.2. Biodistribution Studies

We compared two conjugates, 2.4 CHX-A''/MORAb-009 and 5.5 CHX-A''/MORAb-009 and found differences in the tumor targeting pharmacokinetics between these two conjugates. The conjugate with 2.4 CHX-A'' when co-injected with 30 μ g of MORAb-009 showed a higher tumor uptake (29.8 ± 7.9 vs $15.6 \pm 10.2\%$ ID/g, $p = 0.069$), and a lower liver (10.1 ± 3.4 vs $20.5 \pm 2.9\%$ ID/g, $p < 0.001$) and spleen uptake (13.9 ± 6.7 vs $30.0 \pm 7.7\%$ ID/g, $p < 0.01$) than 5.5 CHX-A'' conjugate at 1 day (Figs. 3a and 3b) when the biodistribution studies were compared in mice with < 200 mg tumors. Since the conjugate with 2.4 CHX-A'' molecules showed a better tumor targeting pharmacokinetics, we used this conjugate to investigate the effect of the dose of unlabeled MORAb-009 on the tumor and organ uptake to find an optimal dose to neutralize the shed-antigen, thereby producing higher tumor-to-organ ratios of ^{111}In -CHX-A''-MORAb-009. In this study, we investigated the effects of three different doses, 0.2, 2, and 30 μ g, of unlabeled MORAb-009 co-injected with ^{111}In -CHX-A''-MORAb-009 and tumor size on the tumor, liver and spleen uptake of ^{111}In -CHX-A''-MORAb-009. The results indicate that the tumor uptake (% ID/g) was greatly affected by the MORAb-009 dose and tumor size (Figs. 3 and 4a). In general, a larger MORAb-009 dose and a smaller tumor size produced a higher tumor uptake. Comparing the tumor uptake of 0.2 and 2 μ g with 30 μ g of unlabeled MORAb-009, co-

injected with ^{111}In -CHX-A''-MORAb-009, the 0.2 and 2 μg MORAb-009 dose produced a lower tumor uptake and retention ($17.6 \pm 4.2\%$ and $10.7 \pm 13.0\%$ ID/g tumor at 1 and 5 day for the 2 μg dose) than the 30 μg injection ($29.8 \pm 7.9\%$ and $39.7 \pm 13.4\%$ ID/g at 1 and 5 day) (Figs. 3a, 3c and 3d) when the tumor uptakes were compared in mice with tumor sizes < 200 mg. We then compared the effect of tumor size up to ~ 400 mg on the tumor, liver and spleen uptake of ^{111}In -CHX-A''-MORAb-009 coinjected with 30 μg MORAb-009. We found that the tumor uptake (% ID/g) was inversely correlated to tumor size, especially when the tumor uptake was compared at 5 day (Fig. 4a), whereas the liver and spleen uptake were proportionally correlated to tumor size (Figs. 4b and 4c). In a separate experiment, we compared the tumor uptake of ^{111}In -CHX-A''-MORAb-009 in the mesothelin-positive A431/K5 tumor with that in the mesothelin-negative tumor. The results showed that the tumor uptake of ^{111}In -CHX-A''-MORAb-009 (2 $\mu\text{Ci}/0.2 \mu\text{g}$) in the mesothelin-positive A431/K5 tumor was more than 4 times of the uptake in the mesothelin-negative A431 tumor (15.7 ± 2.8 vs $3.1 \pm 0.7\%$ ID/g for the negative control at 1 day) for a 7 day period (Fig 3d), indicating that the uptake of the labeled MORAb-009 in the mesothelin-positive A431/K5 tumor is mesothelin-mediated.

4. DISCUSSION

The aims of this study were to optimize the level of CHX-A'' conjugation to MORAb-009 and the injection dose of unlabeled MORAb-009 to obtain high tumor-to-non-tumor uptake ratios of ^{111}In -CHX-A''-MORAb-009 in mice bearing mesothelin-expressing A431/K5 tumors. Since the concentration of mAb in radioimmunoconjugates is often in the range of micromolar concentrations, it would be preferable to conjugate a high number of CHX-A''/MORAb-009 to increase the ^{111}In labeling efficiency and shorten the time required for the completion of the radiolabeling reaction. However, the modification of mAb with the bifunctional chelator can alter the immunoreactivity as well as the solubility of mAb by changing the isoelectric point (pI), thereby affecting the tumor targeting pharmacokinetics. In this study, we conjugated three different numbers (2.4, 3.5, and 5.5) of CHX-A'' molecules to MORAb-009 to investigate the effects of the CHX-A'' conjugation on the ^{111}In labeling efficiency, physical, immunological and pharmacokinetic properties, thereby defining an optimum balance between the radiolabeling efficiency and altered immunoreactivity and pharmacokinetic properties. We first investigated changes in the physical property of the conjugates using size exclusion HPLC, SDS-PAGE (Fig. 1) and isoelectric focusing (Fig. 2). We found that the isoelectric focusing method was the most sensitive to differentiate the conjugates with different CHX-A'' levels; the major band was centered at a pI of ~8.3, ~8.3~7.6, ~7.3, and ~6.3 for MORAb-009 without CHX-A'', the conjugate with 2.4 CHX-A'', 3.5 CHX-A'', and 5.5 CHX-A'', respectively. This finding indicates that the conjugation of CHX-A'' adds a net negative charge to MORAb-009. Among the three conjugates, we selected two conjugates, 2.4 and 5.5 CHX-A''/MORAb-009 with their pIs higher and lower than the physiological pH 7.4 to investigate radiolabeling efficiency, immunoreactivity, and biodistribution. We did not select the conjugate with 3.5 CHX-A'' with a pI of ~7.3 for further studies because this conjugate would become neutral in its net charge at the physiological pH, reducing solubility when used *in vivo*.

We found that conjugates with either 2.4 or 5.5 CHX-A'' molecules resulted in a similarly high ^{111}In labeling yield (> 94%) when performed at a MORAb-009 concentration of 1.0 mg/ml (Table 1). However, the difference in the conjugation level affected the immunoreactivity (Table 1) and the pharmacokinetics (Fig. 3). MORAb-009 is composed of 2 light chains and 2 heavy chains linked together by disulfide bridges. Each light chain has 213 amino acids with 15 lysine residues and each heavy chain has 449 amino acids with 36 lysine residues. There are 3 complementarity-determining regions (CDRs) within the

variable regions of MORAb-009 that bind to antigenic determinants. There are 2 lysine residues from a light chain and 1 lysine residue from a heavy chain involved in these CDRs. We believe that the conjugation of a lysine residue in CDRs with a CHX-A'' molecule completely deactivates the antigen-binding ability of the antibody. Since the number of the lysine residues in CDRs is 5.9% of the total lysine residues (3/51 lysine residues), the probability of deactivating the antibody immunoreactivity is therefore 5.9% if only one CHX-A'' molecule is conjugated to a single MORAb-009. Our tumor cell-binding experiments showed that the immunoreactivity of MORAb-009 with 2.4 CHX-A'' molecules and MORAb-009 with 5.5 CHX-A'' molecules was 88% and 81%, respectively (Table 1). The immunoreactivity (88%) of the 2.4 CHX-A'' conjugate closely reflects the probability of CHX-A'' molecule reacting with the lysine residues in CDRs for the 2.4 CHX-A'' conjugate ($5.9\% \times 2.4 \text{ CHX-A''} = 14\%$ probability of deactivation). However, the immunoreactivity (81%) of the 5.5 CHX-A'' conjugate was larger than the theoretical estimation of immunoreactivity based on the probability of CHX-A'' molecule reacting with the lysine residues in CDRs for the 5.5 CHX-A'' conjugate ($5.9\% \times 5.5 \text{ CHX-A''} = 32\%$ probability of deactivation). This finding implies that the lysine residues in CDRs are somewhat less reactive than the lysine residues in non CDR regions, perhaps due to a steric hindrance especially when a larger number (5.5 CHX-A'' molecules) of a bulky CHX-A'' is conjugated and that the conjugation of CHX-A'' molecules to lysine residues apart from CDRs does not appreciably contribute to the deactivation of MORAb-009.

The results of the biodistribution studies also indicate that the higher conjugation level (5.5 CHX-A'' molecules) produced higher liver and spleen uptake, whereas it produced lower tumor uptake and lower blood pool radioactivity than the 2.4 CHX-A'' conjugate (Fig. 3a). Although this study was not aimed at investigating the mechanism of the high liver and spleen uptake, our finding suggests that a high level (5.5) of conjugation with the negatively charged bulky CHX-A'' could cause a conformational change to mAb that can be recognized by the reticulo-endothelial system in liver and spleen, thereby resulting in rapid sequestration of the radio-conjugate into liver and spleen. It is not likely that the high liver and spleen uptake of the 5.5 CHX-A'' conjugate was resulted from a charge attraction between the radiolabel and the negatively charged phospholipid bi-layers of parenchymal cells of the organs because the conjugation with 5.5 CHX-A'' molecules lowered the pI of MORAb-009 to less than 7 which makes the conjugate negatively charged at the physiological pH, thus causing a charge repulsion with the negatively charged phospholipid bi-layer. The rapid sequestration of the radiolabel into the liver and spleen lowered the blood concentration of the radiolabel from the 5.5 CHX-A'' conjugate which in turn, lowered the tumor uptake of the radiolabel. It is important, therefore, to find an optimal level of CHX-A'' conjugation to achieve a high tumor uptake and a low background radioactivity without compromising the radiolabeling efficiency. These results are similar to those of Al-Ejeh, et al. who demonstrated that mAbs conjugated with DOTA had reduced immunoreactivity and *in vivo* tumor uptake whereas the uptake in liver and spleen increased as the DOTA/mAb ratio increased [30].

Mesothelin is commonly shed into the circulation, and its concentration is correlated with tumor size [21]. Since shed-mesothelin in the circulation can act as a decoy to tumor targeting of mesothelin-specific mAb, it is important to find an optimum dose of MORAb-009 to saturate the circulating antigen and achieve a good tumor targeting dose of $^{111}\text{In-CHX-A''-MORAb-009}$. To determine the optimal MORAb-009 dose, $^{111}\text{In-CHX-A''-MORAb-009}$ with 2.4 CHX-A'' was selected because it showed a higher immunoreactivity, and a better tumor targeting pharmacokinetics than the 5.5 CHX-A'' conjugate. We compared the biodistribution of $^{111}\text{In-CHX-A''-MORAb-009}$ coinjected with 0.2, 2 or 30 μg of a total cold MORAb-009 dose. The results of biodistribution study indicate that the tumor uptake (% ID/g) was greatly affected by the MORAb-009 dose and

tumor size (Figs. 3 and 4a). In general, a larger MORAb-009 dose and a smaller tumor size produced a higher tumor uptake. Comparing the tumor uptake of 0.2 μg and 2 μg with 30 μg of unlabeled MORAb-009 co-injected with $^{111}\text{In-CHX-A''-MORAb-009}$ in mice with a mean tumor size < 200 mg, the 0.2 and 2 μg MORAb-009 doses produced a lower tumor uptake and retention ($17.6 \pm 4.2\%$ and $10.7 \pm 13.0\%$ ID/g tumor at 1 and 5 day, respectively for the 2 μg) of the radiolabel than the 30 μg dose ($29.8 \pm 7.9\%$ and $39.7 \pm 13.4\%$ ID/g at 1 and 5 day) (Figs. 3a, 3c and 3d). Comparatively, the 0.2 μg dose produced higher liver uptake whereas it produced lower tumor uptake and lower blood pool retention of the radiolabel. This finding appears to imply that a large portion of the injected MORAb-009 dose (0.2 μg) was lost to the shed-mesothelin in the circulation, and the $^{111}\text{In-mAb/}$ mesothelin complex was then rapidly sequestered into the liver, lowering the blood pool concentration of $^{111}\text{In-CHX-A''-MORAb-009}$ which in turn, lowered the tumor uptake of the 0.2 μg dose compared to the 30 μg dose. Our finding, that the 30 μg MORAb-009 injection produced high tumor uptake and retention with low liver and spleen uptake, indicates that 30 μg MORAb-009 dose could neutralize shed-mesothelin in the circulation and the interstitial space of tumor in mice with < 200 mg tumors and allow a larger percentage of the radiolabeled mAb to reach the mesothelin sites on the tumor cells. It is also interesting to note that comparing 2 μg with 30 μg dose, these two doses produced similar the liver uptake ($P > 0.05$), but showed different tumor uptake profiles; the 30 μg dose injection not only produced higher tumor uptake values than the 2 μg dose for a 5-day study ($P < 0.05$), but also gradually increased the tumor uptake value up to 5 day whereas the 2 μg dose produced the peak tumor uptake at 1 day, thereafter, gradually decreasing over time. These findings appear to indicate that the 2 μg dose is enough to neutralize the shed-mesothelin in the circulation, but not sufficient to completely neutralize the shed-mesothelin in the interstitial space. The tumor uptake profile for the 2 μg dose suggest that the $^{111}\text{In-CHX-A''-MORAb-009}$ bound to the shed-mesothelin in the interstitial space was not available for binding to the mesothelin on tumor cell surface and this antibody-antigen complex in the interstitial space gradually cleared out of the tumor over time.

We also demonstrated that the tumor uptake of $^{111}\text{In-CHX-A''-MORAb-009}$ in A431/K5 tumor was the mesothelin-specific uptake by comparing with its uptake in the mesothelin-negative A431 tumor. The comparative biodistribution study showed that the tumor uptake of $^{111}\text{In-CHX-A''-MORAb-009}$ (2 $\mu\text{Ci}/0.2 \mu\text{g}$) in the mesothelin-positive A431/K5 tumor was more than 4 times of the uptake in the mesothelin-negative A431 tumor for a 7 day period (inset of Fig 3d), indicating that the uptake of the labeled MORAb-009 in the mesothelin-positive A431/K5 tumor was mesothelin-mediated.

In conclusion, this study suggests that the level of chelator conjugation to mAb affects the integrity of the physical, immunological, and pharmacokinetic properties of mAb. In addition, this study also suggests that it may be necessary to adjust the co-injected dose of unlabeled MORAb-009 to counteract shed-antigen concentration in the circulation and interstitial space to achieve a high tumor uptake while reducing liver and spleen uptake of the radiolabeled MORAb-009.

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References

1. Carrasquillo JA, Krohn KA, Beaumier P, McGuffin RW, Brown JP, Hellstrom KE, et al. Diagnosis of and therapy for solid tumors with radiolabeled antibodies and immune fragments. *Cancer Treat Rep.* 1984; 68:317–28. [PubMed: 6607111]

2. Mulligan T, Carrasquillo JA, Chung Y, Milenic DE, Schlom J, Feuerstein I, et al. Phase I study of intravenous Lu-labeled CC49 murine monoclonal antibody in patients with advanced adenocarcinoma. *Clin Cancer Res.* 1995; 1:1447–54. [PubMed: 9815943]
3. Waldmann TA, White JD, Carrasquillo JA, Reynolds JC, Paik CH, Gansow OA, et al. Radioimmunotherapy of interleukin-2R alpha-expressing adult T-cell leukemia with Yttrium-90-labeled anti-Tac. *Blood.* 1995; 86:4063–75. [PubMed: 7492762]
4. Carrasquillo JA, White JD, Paik CH, Raubitschek A, Le N, Rotman M, et al. Similarities and differences in ¹¹¹In- and ⁹⁰Y-labeled 1B4M-DTPA antiTac monoclonal antibody distribution. *J Nucl Med.* 1999; 40:268–76. [PubMed: 10025834]
5. Pai-Scherf LH, Carrasquillo JA, Paik C, Gansow O, Whatley M, Pearson D, et al. Imaging and phase I study of ¹¹¹In- and ⁹⁰Y-labeled anti-LewisY monoclonal antibody B3. *Clin Cancer Res.* 2000; 6:1720–30. [PubMed: 10815890]
6. Kaminski MS, Zelenetz AD, Press OW, Saleh M, Leonard J, Fehrenbacher L, et al. Pivotal study of iodine I-131 tositumomab for chemotherapy-refractory low-grade or transformed low-grade B-cell non-Hodgkin's lymphomas. *J Clin Oncol.* 2001; 19:3918–28. [PubMed: 11579112]
7. Witzig TE, Gordon LI, Cabanillas F, Czuczman MS, Emmanouilides C, Joyce R, et al. Randomized controlled trial of yttrium-90-labeled ibritumomab tiuxetan radioimmunotherapy versus rituximab immunotherapy for patients with relapsed or refractory low-grade, follicular, or transformed B-cell non-Hodgkin's lymphoma. *J Clin Oncol.* 2002; 20:2453–63. [PubMed: 12011122]
8. DeNardo GL, Sysko VV, DeNardo SJ. Cure of incurable lymphoma. *Int J Radiat Oncol Biol Phys.* 2006; 66:S46–56. [PubMed: 16979440]
9. Meares CF. Chelating agents for the binding of metal ions to antibodies. *Int J Rad Appl Instrum B.* 1986; 13:311–8. [PubMed: 3098704]
10. Kozak RW, Raubitschek A, Mirzadeh S, Brechbiel MW, Junghans RP, Gansow OA, et al. Nature of the bifunctional chelating agent used for radioimmunotherapy with yttrium-90 monoclonal antibodies: critical factors in determining in vivo survival and organ toxicity. *Cancer Res.* 1989; 49:2639–44. [PubMed: 2785435]
11. Deshpande SV, DeNardo SJ, Kukis DL, Moi MK, McCall MJ, DeNardo GL, et al. Yttrium-90-labeled monoclonal antibody for therapy: labeling by a new macrocyclic bifunctional chelating agent. *J Nucl Med.* 1990; 31:473–9. [PubMed: 2324823]
12. Camera L, Kinuya S, Garmestani K, Pai LH, Brechbiel MW, Gansow OA, et al. Evaluation of a new DTPA-derivative chelator: comparative biodistribution and imaging studies of ¹¹¹In-labeled B3 monoclonal antibody in athymic mice bearing human epidermoid carcinoma xenografts. *Nucl Med Biol.* 1993; 20:955–62. [PubMed: 8298575]
13. Camera L, Kinuya S, Garmestani K, Wu C, Brechbiel MW, Pai LH, et al. Evaluation of the serum stability and in vivo biodistribution of CHX-DTPA and other ligands for yttrium labeling of monoclonal antibodies. *J Nucl Med.* 1994; 35:882–9. [PubMed: 8176477]
14. Pham DT, Kaspersen FM, Bos ES. Electrophoretic method for the quantitative determination of a benzyl-DTPA ligand in DTPA monoclonal antibody conjugates. *Bioconjug Chem.* 1995; 6:313–5. [PubMed: 7632804]
15. Lewis MR, Kao JY, Anderson AL, Shively JE, Raubitschek A. An improved method for conjugating monoclonal antibodies with N-hydroxysulfosuccinimidyl DOTA. *Bioconjug Chem.* 2001; 12:320–4. [PubMed: 11312695]
16. Paik CH, Ebbert MA, Murphy PR, Lassman CR, Reba RC, Eckelman WC, et al. Factors influencing DTPA conjugation with antibodies by cyclic DTPA anhydride. *J Nucl Med.* 1983; 24:1158–63. [PubMed: 6644376]
17. Paik CH, Murphy PR, Eckelman WC, Volkert WA, Reba RC. Optimization of the DTPA mixed-anhydride reaction with antibodies at low concentration. *J Nucl Med.* 1983; 24:932–6. [PubMed: 6619963]
18. Kukis DL, DeNardo GL, DeNardo SJ, Mirick GR, Miers LA, Greiner DP, et al. Effect of the extent of chelate substitution on the immunoreactivity and biodistribution of 2IT-BAT-Lym-1 immunoconjugates. *Cancer Res.* 1995; 55:878–84. [PubMed: 7850803]
19. Knogler K, Grunberg J, Novak-Hofer I, Zimmermann K, Schubiger PA. Evaluation of ¹⁷⁷Lu-DOTA-labeled aglycosylated monoclonal anti-L1-CAM antibody chCE7: influence of the number

- of chelators on the in vitro and in vivo properties. *Nucl Med Biol.* 2006; 33:883–9. [PubMed: 17045168]
20. Chang K, Pastan I, Willingham MC. Isolation and characterization of a monoclonal antibody, K1, reactive with ovarian cancers and normal mesothelium. *Int J Cancer.* 1992; 50:373–81. [PubMed: 1735605]
21. Chang K, Pastan I. Molecular cloning of mesothelin, a differentiation antigen present on mesothelium, mesotheliomas, and ovarian cancers. *Proc Natl Acad Sci U S A.* 1996; 93:136–40. [PubMed: 8552591]
22. Hassan R, Ebel W, Routhier EL, Patel R, Kline JB, Zhang J, et al. Preclinical evaluation of MORAb-009, a chimeric antibody targeting tumor-associated mesothelin. *Cancer Immun.* 2007; 7:20. [PubMed: 18088084]
23. Hassan R, Cohen SJ, Phillips M, Pastan I, Sharon E, Kelly RJ, et al. Phase I Clinical Trial of the Chimeric Anti-Mesothelin Monoclonal Antibody MORAb-009 in Patients with Mesothelin Expressing Cancers. *Clinical Cancer Research.* 2011 in press.
24. Hassan R, Remaley AT, Sampson ML, Zhang J, Cox DD, Pingpank J, et al. Detection and quantitation of serum mesothelin, a tumor marker for patients with mesothelioma and ovarian cancer. *Clin Cancer Res.* 2006; 12:447–53. [PubMed: 16428485]
25. Zhang Y, Xiang L, Hassan R, Pastan I. Immunotoxin and Taxol synergy results from a decrease in shed mesothelin levels in the extracellular space of tumors. *Proc Natl Acad Sci U S A.* 2007; 104:17099–104. [PubMed: 17940013]
26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72:248–54. [PubMed: 942051]
27. Pippin CG, Parker TA, McMurry TJ, Brechbiel MW. Spectrophotometric method for the determination of a bifunctional DTPA ligand in DTPA-monoclonal antibody conjugates. *Bioconj Chem.* 1992; 3:342–5. [PubMed: 1390990]
28. Lindmo T, Bunn PA Jr. Determination of the true immunoreactive fraction of monoclonal antibodies after radiolabeling. *Methods Enzymol.* 1986; 121:678–91. [PubMed: 3523136]
29. Hassan R, Wu C, Brechbiel MW, Margulies I, Kreitman RJ, Pastan I. ¹¹¹Indium labeled monoclonal antibody K1: biodistribution study in nude mice bearing a human carcinoma xenograft expressing mesothelin. *Int J Cancer.* 1999; 80:559–563. [PubMed: 9935157]
30. Al-Ejeh F, Darby JM, Thierry B, Brown MP. A simplified suite of methods to evaluate chelator conjugation of antibodies: effects on hydrodynamic radius and biodistribution. *Nucl Med Biol.* 2009; 36:395–402. [PubMed: 19423007]

Fig. 1. SDS-PAGE electrophoresis of CHX-A''-MORAb-009 conjugates with different levels of CHX-A'' conjugation. Lanes: (M1 and M2) Molecular Weight Marker; (1) Unconjugated MORAb-009; (2) 2.4 CHX-A''/MORAb-009; (3) 3.5 CHX-A''/MORAb-009; (4) 5.5 CHX-A''/MORAb-009; (5) Unconjugated MORAb-009 (reduced form); (6) 2.4 CHX-A''/MORAb-009 (reduced form); (7) 3.5 CHX-A''/MORAb-009 (reduced form); (8) 5.5 CHX-A''/MORAb-009 (reduced form). The samples in the reduced form were obtained by treatment with 2-mercaptoethanol which reduced the interchain disulfide bridges. All 3 conjugates showed the electrophoretic mobility similar to that of the unconjugated mAb.

Fig. 2. Isoelectric focusing of CHX-A''-MORAb-009 with different levels of CHX-A'' conjugation. Lanes: (1 and 6) pI Marker; (2) Unconjugated MORAb-009; (3 and 7) 2.4 CHX-A''/MORAb-009 conjugated in duplicate; (4 and 8) 3.5 CHX-A''/MORAb-009 conjugated in duplicate; (5 and 9) 5.5 CHX-A''/MORAb-009 conjugated in duplicate. The pI of the conjugates decreased as the CHX-A'' conjugation level increased.

Fig. 3.

Effect of CHX-A'' conjugation level and injection dose on biodistribution of ^{111}In -CHX-A''-MORAb-009 in nude mice with tumor sizes < 200 mg. The biodistribution data from ^{111}In -CHX-A''-MORAb-009 (2 $\mu\text{Ci}/30 \mu\text{g}$) with 2.4 CHX-A''/MORAb-009 (n = 6-14) and 5.5 CHX-A''/MORAb-009 (n = 5) are compared in Figs. 3a and 3b, respectively. The biodistribution data from the ^{111}In -CHX-A''-MORAb-009 with three different injected doses, (2 $\mu\text{Ci}/30 \mu\text{g}$), (2 $\mu\text{Ci}/2 \mu\text{g}$), and (2 $\mu\text{Ci}/0.2 \mu\text{g}$) are compared in Figs. 3a, 3c, and 3d, respectively. The analysis of statistical significance in organ uptake data: * $P < 0.001$, ** $0.001 < P < 0.01$, *** $0.01 < P < 0.05$; † $P < 0.001$, †† $0.001 < P < 0.01$; ‡ $P < 0.001$, ‡‡ $0.001 < P < 0.01$, ‡‡‡ $0.01 < P < 0.05$; # $P < 0.001$, ## $0.001 < P < 0.01$. Column, mean; bar, S.D. The 2.4 CHX-A'' conjugate produced a higher tumor uptake, but a lower liver and spleen uptake than the 5.5 CHX-A'' conjugate. The 30 μg dose produced a higher tumor uptake than the 0.2 and 2 μg doses, and a lower liver uptake than the 0.2 μg dose.

Fig. 4. Effect of tumor size on tumor, liver, and spleen uptake of ^{111}In -CHX-A''-MORAb-009 with 2.4 CHX-A'' (2 μCi /30 μg MORAb-009). (a) Tumor uptake vs tumor size; (b) Liver uptake vs tumor size; (c) Spleen uptake vs tumor size. The tumor uptake was inversely correlated with tumor size whereas the liver and spleen uptakes were positively correlated with tumor size.

Table 1Description of CHX-A''-MORAb-009 and ^{111}In -CHX-A''-MORAb-009

	Conjugation Level (CHX-A'' /MORAb-009)	Isoelectric Point (pI)	Labeling Yield (%)	Immunoreactivity* (%)
MORAb	-	~8.3	-	-
A	2.4 $\pm$ 1.1 (n=3)	~8.3~7.6	95.2 (n=2)	88.3 $\pm$ 4.5 (n= 8)
Conjugates B	3.5	~7.3	-	-
C	5.5 $\pm$ 1.6 (n=3)	~6.3	94.8 (n=2)	81.1 $\pm$ 0.9 (n = 6)

* $P < 0.05$