Biodistribution Study of $^{188}$Re-Labeled Trisuccin-HuCC49 and Trisuccin-HuCC49ΔCh2 Conjugates in Athymic Nude Mice Bearing Intraperitoneal Colon Cancer Xenografts

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Abstract

The trihydroxamate bifunctional chelating agent (BCA), trisuccin, has been shown to be a potential ligand for radiolabeling of monoclonal antibodies (MAbs) with rhenium radioisotopes, through an indirect postconjugation approach. The use of this trihydroxamate BCA made it possible to prepare stable BCA-MAb conjugates in pure form that could be radiolabeled with carrier-free $^{188}$Re. The anti-TAG-72 murine MAb, CC49, and its humanized derivatives are promising agents in the treatment of a number of malignancies with the Cu²⁺ domain-deleted MAb (HuCC49ΔCh2), which is of particular interest due to its rapid blood clearance. The biodistribution of $^{188}$Re-labeled conjugates of trisuccin with both humanized CC49 (HuCC49) and HuCC49ΔCh2 in athymic nude mice implanted i.p. with LS174T human colon carcinoma was studied. Trisuccin-MAb conjugates were synthesized at different BCA:MAb ratios by the 6-oxoheptanoic acid method using trisuccin hydrazide. The conjugates were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for the number of incorporated trisuccin molecules. The conjugates were radiolabeled with carrier-free, generator-produced $^{188}$Re and purified by gel filtration on Sephadex G-25. Labeling yields and homogeneity of the labeled conjugates were analyzed by high-pressure liquid chromatography and instant TLC. Athymic nude mice were injected i.p. with LS174T human colon carcinoma cells, 7 days prior to injection of the labeled antibodies. $^{188}$Re-labeled MABs were injected i.p., and the mice were sacrificed 24 h postinjection. Matrix-assisted laser desorption/ionization time-of-flight analyses showed stable incorporation of trisuccin into each MAB, with the measured ligand:MAb values positively correlating with the theoretical ratios. Labeling of the conjugates with $^{188}$Re proceeded with high yields, producing homogeneous $^{188}$Re-MAbs with good stabilities as shown by instant TLC and biodistribution analyses. Biodistribution of the radiolabeled MAbs at 24 h after injection showed median tumor uptake values of 23.5% ID/g and 17.6% ID/g for the $^{188}$Re-HuCC49ΔCh2 and $^{188}$Re-HuCC49, respectively. The blood clearance of the domain-deleted MAb was faster than that of the intact antibody. The blood values at 24 h after injection were 0.7% ID/g for $^{188}$Re-HuCC49ΔCh2 and 3.2% ID/g for $^{188}$Re-HuCC49. The results indicate that trisuccin is a promising agent for postconjugation labeling of antibodies with $^{188}$Re. Additionally, these results illustrate the potential of $^{188}$Re-HuCC49ΔCh2 in radioimmunodiagnostics and radioimmunotherapy of cancer.

Introduction

Radiolabeled MAbs have shown promise for cancer therapy (1–8). Although utilization of this approach was started with isotopes of iodine as the source of radioactivity (9–15), it was realized later that the labeling of MAbs with radioisotopes might offer advantages (16–19). These include lack of dehalogenation, higher tumor uptake, and longer retention in tumor. The indirect labeling method, in which a BCA serves as a linker between the antibody and the radiometal, may offer chemical and biological advantages due to better-defined metal chelate structures and higher immunoreactivities (20, 21). In the postconjugation radiolabeling techniques, the BCA is first conjugated to the antibody and the resulting conjugate is then radiolabeled in a second step.

Radioisotopes of the transition metal rhenium (i.e., $^{186}$Re and $^{188}$Re) have physical properties that may be useful in the radioimmunodiagnostics and RIT of cancer (22–25). $^{188}$Re is produced by neutron bombardment of the stable $^{186}$Re leading to a low specific activity product, whereas $^{188}$Re is available carrier-free from a $^{188}$W/$^{188}$Re generator (23). In addition to generating β-particles with suitable energies for therapy, these radioisotopes also emit γ-rays with energies suitable for imaging. Whereas $^{188}$Re, with a half-life of 90 h, may be appropriate for antibodies with long circulation times, $^{188}$Re with a half-life of 17 h, may be suitable for smaller molecules, such as antibody fragments and derivatives.

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The abbreviations used are: MAB, monoclonal antibody; BCA, bifunctional chelating agent; L:MAb ratio, ligand:MAb ratio; OHA, 6-oxoheptanoic acid; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; HPLC, high-pressure liquid chromatography; RIT, radioimmunotherapy; DPBS, Dulbecco’s PBS; MW, molecular weight; SEC, size-exclusion chromatography; %ID/g, percentage of injected dose per gram of tissue; RY, radiolabeling yield; ITLC, instant TLC.
Despite their attractive features, the chelation chemistries of rhenium isotopes have hampered their full utilization in radioimmunodiagnosis and RIT. In contrast to $^{99m}$Tc, $^{186/188}$Re isotopes are difficult to reduce to lower oxidation states, a process necessary for their ligation by organic BCAs (26). Higher concentrations of the reducing agent (e.g., SnCl$_2$) at elevated temperatures and longer reaction times are required for radiolabeling with $^{186/188}$Re isotopes. A rhenium-specific BCA must also have appropriate features to satisfy the requirements of stable antibody conjugation. Most importantly, the metal-chelating groups of this compound must not interfere chemically with the antibody binding terminus.

Of different BCAs examined for $^{186/188}$Re and $^{99m}$Tc-labeling of MAbs, only the amido-thiol system has been in frequent use (27-29). These molecules consist of one or two sulfur atoms in its sulfide form and two or three nitrogen atoms, usually as amide groups. These produce stable ligands for both $^{186/188}$Re and $^{99m}$Tc radionuclides. However, because of the reactivity of the thiol groups, the standard procedure of post-conjugation radiolabeling used for other rheniometals is not feasible or, at best, not efficient. Interference from the thiol functions necessitates masking the BCA prior to conjugation to the MAb. Because removal of the thiol protecting groups is not chemically compatible with maintaining the biological integrity of the antibody, the thiol masking and radiometal chelation have been combined in a procedure referred to as the “preformed chelate approach” (27, 30). In this protocol, the activated BCA is first labeled at high temperature with $^{186/188}$Re or $^{99m}$Tc isotopes, in the presence of stannous ion. In a second step, the complex is purified on a reversed-phase cartridge, and the pH of the medium is adjusted to a pH suitable for antibody conjugation. Next, the purified activated chelate is conjugated to the antibody. A second purification affords the final labeled antibody. Low to medium RYs are achieved.

In an effort to design rhenium-binding BCAs without the shortcomings discussed above, we introduced hydroxamic acids as a new class of BCAs and reported on the synthesis of the first member of this family, trisuccin (31). In this design, we were interested in a chelator that allows the postconjugation radiolabeling of antibodies with isotopes of $^{186/188}$Re or $^{99m}$Tc isotopes, in the presence of stannous ion. In a second step, the complex is purified on a reversed-phase cartridge, and the pH of the medium is adjusted to a pH suitable for antibody conjugation. Next, the purified activated chelate is conjugated to the antibody. A second purification affords the final labeled antibody. Low to medium RYs are achieved.

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MAb ratio of 7.5, in 50 mM PBS buffer at pH 8.1 and at 0°C for 1 h. The conjugate was purified and buffer-exchanged in a Centricon concentrator (Amicon, Beverly, MA) with 50 mM acetate buffer at pH 5.5. For each antibody, this solution was divided into three 300-ml portions for the trisuccin conjugation (Step 2).

**Antibody Conjugation. Step 2: Trisuccin-MAb.** To the solution of the OHA-MAb from Step 1, and at 0°C, a solution of the trisuccin hydrazide was added followed by NaCNBH3 after 1 h and at an overall concentration of 50 mM. Three trisuccin-MAb molar ratios (100:1, 300:1, and 500:1) were used for each MAb. The mixture was stirred at 4°C for 18 h and purified as above.

**MALDI-TOF Analyses.** Samples were analyzed in the positive mode on a Voyager Elite mass spectrometer with delayed extraction technology (PerSeptive Biosystems). The acceleration voltage was set at 25 kV, and 50–100 laser shots were summed. Sinapinic acid (Aldrich), dissolved in a 1:1 (v/v) mixture of acetonitrile and 0.1% trifluoroacetic acid, was used as the matrix. A 1 pmol/µl solution of BSA was added as an internal standard. Equal volumes of sample and matrix were mixed on a smooth plate. For each reaction, the intact MAb was scanned as the control for evaluation of the conjugation. The average of three measurements was used. For the OHA method, the MW increase of the OHA-MAb intermediate was calculated using the unconjugated antibody. The MW of the trisuccin conjugates (41) were added to other tubes. To all tubes were added 100 µl of the labeled MAb (12,000–20,000 cpm, approximately 10 mg/ml), and HuCC49ACH2 were 9.1 and 9.5 min, respectively, for the UV traces followed, within about 0.4 min, by the radioactive traces.

Immunoactivity Assay. A modified Lindmo assay was used to evaluate the immunoactivity of the radiolabeled conjugates (41). Briefly, polystyrene beads (6.4 mm, specular finish) were coated with TAG-72 positive bovine submaxillary mucin (Sigma Chemical Co., St. Louis, MO) in PBS (4.4 mg/100 ml) and blocked with 0.1% human serum albumin. A control test tube containing no bead, as a counting standard for added 188Re, and a nonspecific binding tube containing the bead and 5 µl of a 5 mg/ml solution of the unlabeled MAb, as a competitor of the labeled MAb, were used. Five µL of decreasing concentrations (1:2 to 1:200) of the same unlabeled MAb were added to other tubes. To all tubes were added 100 µl of the labeled MAb (12,000–20,000 cpm, approximately 10 mg/ml), which were vortexed at room temperature for 1 h. All tubes, excluding the counting standard, were washed with DPBS, and the beads were transferred into clean test tubes and counted in a gamma counter. The percentage of radioactivity bound to the mucin coated beads was calculated. The inverse of the percentage of binding values was plotted against the cold MAb concentration. Extrapolation to infinite MAb concentration determined the percentage of immunoactivity of the labeled MAbs.

**Animal Model.** Athymic nude female nu/nu mice with a BALB/c background, were obtained from the National Cancer Institute-Frederick Cancer Research & Development Center (Frederick, MD) and were kept under sterile conditions. Procedures to minimize discomfort, pain, and distress were in accord with the Animal Resource Program at the University of Alabama at Birmingham, accredited by the American Association for Accreditation of Laboratory Animal Care. The LS174T human colon cancer cells (American Type Culture Collection, Manassas, VA) were harvested and suspended in sterile PBS at a concentration of 7.5×10^6 viable cells/ml. Cell viability was determined by trypan blue dye exclusion. Viable cells (10^5) in sterile PBS were injected i.p. into the mice.

**Biodistribution of 188Re-MAb Conjugates.** Groups of mice with established 7-day i.p. tumors received 5 µCi of the 188Re-labeled antibodies as an i.p. bolus injection. At 24 h
postinjection, the animals were exsanguinated after anesthesia and dissected for determination of biodistribution. Samples of blood, heart, lung, liver, stomach, small intestine, spleen, kidney, skin, bone, muscle, lining of the abdominal cavity, uterus, pancreas, and tumor were blotted dry, weighed, and counted for the uptake of radioactivity in a well-type gamma counter (Minaxi-gamma 5000 series, Packard, Chicago, IL). The %ID/g for each tissue 24 h postinjection for the HuCC49 and HuCC49ACH2 are summarized by the median and the interquartile range. The tumor-to-tissue ratios 24 h postinjection for lSSRe-HuCC49 and laaRe-HuCC49ACH2 are also summarized by the median and interquartile range. Statistical comparisons of the %ID/g of the tissues as well as the tumor-to-tissue ratios between the 1SSRe_HuCC49 and lSaRe-HuCC49ACH2 were performed using the Wilcoxon rank-sum test.

Results

Synthesis of Trisuccin and Antibody Conjugation. The trisuccin hydroxamate was obtained in 72% overall chemical yield and >98% purity as determined by 1H NMR and TLC analyses. The OHA-MAb conjugate was synthesized at a 7.5:1 theoretical OHA:MAb molar ratio, whereas trisuccin was conjugated at theoretical molar ratios of 100, 300, and 500 under identical reaction and purification conditions. The theoretical values represent the measured quantity of trisuccin introduced into the conjugation mixture to achieve the designated molar ratio. The experimental ratios, measured directly by MALDI-TOF, for both the intermediate and final-product conjugates are shown in Table 1.

Radiolabeling of Antibody Conjugates. The conjugates were labeled with reduced 188Re by incubation of the conjugates at 45°C for 45 min. An unconjugated HuCC49ΔCt2 showed 0.25 μCi/μg of nonspecific labeling after 90 min of incubation with reduced 188Re. The conjugates showed immunoreactivities of ≥69%. The radiolabeling and immunoreactivity data are shown in Table 2.

Biodistribution of 188Re-MAb Conjugates. Purified labeled conjugates were injected i.p. into the tumor bearing mice, and the animals were sacrificed at 24 h postinjection. Biodistribution analyses were performed on the dissected organs. These data are shown in Fig. 1. The median tumor concentrations for 188Re-HuCC49 and 188Re-HuCC49ΔCt2 were 17.6%ID/g and 23.5%ID/g, respectively, and did not differ (P = 0.905). Liver and spleen showed relatively high uptake of both conjugates, which is typical for chelate conjugated antibodies. The respective blood concentrations for 188Re-HuCC49 and 188Re-HuCC49ΔCt2 were 5.2 and 0.7%ID/g (P = 0.016). The 188Re-HuCC49ΔCt2 showed a higher uptake in bone and abdominal lining than 188Re-HuCC49. Tumor nodules were adherent to the uterus, which explains the uptake of both conjugates in uterus. The tumor-to-blood ratios for 188Re-HuCC49 and 188Re-HuCC49ΔCt2 were significantly different (P = 0.016; Fig. 2). Other significantly different tumor-to-tissue ratios occurred in kidney, bone, and abdominal lining.

Discussion

The labeling of MAbs with rhenium radioisotopes for RIT has been through two main labeling protocols: the direct method, with binding of the radiometal to endogenous thiol groups, and the indirect approach, using an organic linker as the chelating agent. The direct protocol has been extensively studied for labeling with both 99mTc (42) and 188Re (22, 43). Despite its advantages, the indirect procedure has been limited to only a few BCAs for radiometal complexation. Due to the spontaneous oxidation of unprotected thiols to the corresponding disulfides under the 186/188Re/99mTc labeling conditions, this design of chelating agents imparts lengthy and tedious procedures for their utilization.

During recent years, major progress has been made toward the design and development of a 188Re generator to efficiently produce the high specific activity isotope in ranges useful to...
clinical applications. To this end, Knapp and co-workers (23, 40) at the Oak Ridge National Laboratory have developed and manufactured such a generator with optimized operation and elution protocols. The availability of such a generator has been encouraging for further development of $^{188}$Re-labeling protocols for MAbs.

The BCA trisuccin, introduced by this laboratory as a rhenium chelator, belongs to the family of hydroxamic acids, with known abilities for metal chelation (44—46). The rationale for the selection of hydroxamates was based partly on the oxyphilic nature of rhenium, thus eliminating the need for any sulfur-containing functionality, and the advantage of postconjugation radiolabeling. Because of this, the conjugate between the MAb and the BCA could be prepared, purified, and characterized prior to the rhenium radiolabeling. We recently reported a procedure, termed the OHA method (33), using the low-pH selective coupling of hydrazines with ketone carbonyl groups, for conjugation of trisuccin to antibodies for subsequent rhenium radiolabeling.

From our previous report (33) and the work described here, it is proven that the OHA method efficiently produces stable hydroxamate-antibody conjugates. Notable advantages of this method are the selectivity of the conjugation chemistry, in which the hydrazide function of the BCA binds to the ketone linker (OHA) in the low-pH medium without detectable side reactions with the native amine groups of the antibody (47), and the fact that the OHA linker removes the BCA further from the bulk of the MAb molecule. This selectivity, which is compatible with all reactive functionalities of the antibody, is a significant advantage of this technique. The use of MALDI-TOF also proves to be invaluable for the analysis of the outcome of the conjugation reactions. Unlike the routinely used indirect gel electrophoretic analyses for MW determination of proteins, MALDI-TOF provides a direct and unambiguous means of measurement with high accuracy. Whereas the MW for HuCC49ACH2, as measured by SDS-PAGE, had been reported to be 159,000 (35), our mass spectroscopic direct measurements showed a MW of 121,000. Similarly, for the HuCC49 antibody, our measured MW of 148,000 was lower than the previously reported SDS-PAGE MWs of 160,000 (48) and 169,000 (35).

To select the trisuccin-MAb conjugate with the highest immunoreactivity for biodistribution studies, three conjugates from each antibody, with theoretical trisuccin:MAb molar ratios of 100, 300, and 500, were prepared. Conjugations were started by using a 7.5:1 molar ratio of the OHA linker with respect to each MAb, HuCC49 or HuCC49ACH2. The experimental values as measured by MALDI-TOF were 5.1 and 5.5 for OHA:HuCC49 and OHA:HuCC49ACH2, respectively, indicating rapid and efficient conjugations at a low reaction temperature of 0°C and within 1 h. For the second step, conjugation of trisuccin, the actual degree of substitution increased within a range of only 1.4 trisuccin:MAb, although the molar ratios for the added trisuccin hydrazide reagent varied from 100 to 500 (trisuccin:MAb). This narrow range of L:MAb variation indicates the limiting effect of the OHA anchor and clearly illustrates the specificity of this conjugation process, in which the BCA (trisuccin hydrazide) exclusively targets the ketone functions of the OHA linker without interfering with the native functionalities of the protein.

$^{188}$Re labeling of the conjugates proceeded with high RYs (mean RY = 88.5 ± 7.1%), and a mean specific activity of 2.9 ± 1.2 μCi/μg. The specific activities of the HuCC49 conjugates remained fairly constant, whereas those of the HuCC49ACH2 conjugates increased with an increase in the RY, which could be due to the higher losses of the larger MW protein during the labeling and purification process. Although the immunoreactivities for all six preparations were not widely different (mean immunoreactivity, 71.8 ± 3.3%), we selected the conjugates with the lowest theoretical L:MAb ratio of 100 for in vivo studies.
Biodistribution at 24 h after injection in athymic nude mice bearing i.p. implanted LS174T tumors and inoculated i.p. with 5 μCi of the purified labeled conjugates showed a high median tumor uptake in both groups, i.e., 17.6%ID/g and 23.5%ID/g for $^{188}$Re-HuCC49 and $^{188}$Re-HuCC49ΔCn2, respectively. These tumor concentrations were not significantly different. Slavin-Chiorini et al. (48) reported a lower tumor uptake of iodinated chimeric MAb's cCC49A2c versus cCC49, and $^{177}$Lu-cCC49ΔCn2 and cCC49ΔCn1 versus cCC49 following i.v. injection in animals bearing s.c. LS174T tumors due to the more rapid blood clearance of the domain-deleted antibodies. Slavin-Chiorini et al. (49) have also reported the same 24 h localization pattern in the biodistribution studies of iodinated cB72.3ΔCn2 and cB72.3 antibodies following systemic administration. The lower tumor uptake of a Cn2 domain-deleted MAb has also been reported for the antidisialoganglioside GD2 antibody, 14.18, by Mueller et al. (50) following systemic administration. These results illustrate the advantage of regional i.p. administration of the HuCC49ΔCn2 versus HuCC49 for achieving similar i.p. tumor localization.

The blood levels of the two labeled conjugates showed an expected pattern noted previously by other investigators for the iodinated analogues (38). The HuCC49ΔCn2 conjugate, with a MW about 82% of that for the HuCC49, showed a considerably faster blood clearance. This may have been because of removal of the glycosylation site present in the CH2 region, which may facilitate receptor-mediated clearance through the liver (48). At the 24 h time point, the median blood concentration for the $^{188}$Re-HuCC49 conjugate was 3.2%ID/g, whereas the median blood concentration of the $^{188}$Re-HuCC49ΔCn2 conjugate was 0.7%ID/g. This is in agreement with the findings on the biodistribution of these two antibodies when labeled with either $^{131}$I or $^{125}$I following i.v. or i.p. injection (38). There were differences in uptake of the two conjugates in other normal tissues including kidney, bone, and abdominal lining. The $^{188}$Re-HuCC49ΔCn2 showed a significantly higher tumor-to-blood ratio than $^{188}$Re-HuCC49, and significantly lower tumor-to-kidney, tumor-to-bone, and tumor-to-abdominal lining ratios. The other tumor-to-normal tissue ratios were not significantly different. These results illustrate the potential therapeutic advantage of $^{188}$Re-HuCC49ΔCn2 following regional administration for i.p. cancer.

Conclusion. The high-efficiency labeling of the Cn2 domain-deleted humanized anti-TAG-72 antibody, HuCC49ΔCn2, with $^{188}$Re using the trihydroxamate ligand trisuccin, was accomplished. For a comparative study, the radiolabeling and biodistribution in athymic mice was also carried out with the intact HuCC49. Antibody conjugations were performed with the recently synthesized trisuccin hydrazide and showed excellent consistency and control on the L:MAb ratios. The conjugates underwent postconjugation labeling with $^{188}$Re at high radiochemical yields. The labeled conjugates were injected i.p. into athymic nude mice bearing i.p. LS174T human colon carcinoma xenografts. Biodistribution analyses were performed 24 h postinjection and showed high tumor uptake of both conjugates and rapid clearance of serum activity for the ΔCn2 conjugate. These findings suggest that (a) trisuccin is a good reagent for the postconjugation radiolabeling of antibodies with rhodium isotopes, and (b) $^{188}$Re-HuCC49ΔCn2 is a promising agent for RIT of i.p. cancer due to its favorable immuneactivity, pharmacokinetics, and biodistribution characteristics.

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References


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