Radioimmunotherapy of Human Colon Carcinoma Xenografts Using a ^{213}Bi -Labeled Domain-Deleted Humanized Monoclonal Antibody

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ABSTRACT

The data presented within this paper is the first report of a humanized domain-deleted monoclonal antibody (HuCC49ΔCH2) to be utilized in a radioimmunotherapeutic (RIT) application with ^{213}Bi . An initial study indicated that ^{111}In -HuCC49ΔCH2 targets the subcutaneously implanted human colon carcinoma xenograft, LS-174T, when injected via a peritoneal route. The HuCC49ΔCH2 was then radiolabeled with ^{213}Bi , an α -emitting radionuclide with a half-life of 45.6 minutes, and evaluated for therapeutic efficacy. Dose titration studies indicated that a single dose of 500–1000 μCi , when injected by an intraperitoneal route, resulted in the growth inhibition or regression of the tumor xenograft. The radioimmunotherapeutic effect was found to be dose-dependent. Specificity of the therapeutic efficacy was confirmed in a subsequent experiment with athymic mice bearing TAG-72 negative MIP (human colorectal) xenografts. A preliminary study was also performed to assess a multiple-dose administration of ^{213}Bi -HuCC49ΔCH2. Doses (500 μCi) were administered at 14-day intervals after tumor implantation. A reduction in volume and/or delay in tumor growth was evident following the second and third injections of ^{213}Bi -HuCC49ΔCH2. As further validation of the use of ^{213}Bi -HuCC49ΔCH2 for RIT, a study using ^{131}I was conducted. The overall survival of mice receiving ^{213}Bi -HuCC49ΔCH2 was greater than those that received ^{131}I -HuCC49ΔCH2.

Key words: radioimmunotherapy, α -particle, bismuth, ^{213}Bi , HuCC49ΔCH2

INTRODUCTION

Systemic radiotherapy, the delivery of therapeutic radionuclides to cancer cells via monoclonal antibodies (MAb), remains an attractive, viable

modality for the treatment and management of cancer. The field of radioimmunotherapy (RIT) has regained momentum with successes realized in the treatment of hematopoietic malignancies using ^{90}Y - and ^{131}I -labeled MAb, resulting in FDA-approval of the first ^{90}Y -labeled MAb (Zevalin,TM (Biogen Idec, Cambridge, MA) and anti-CD20 MAb) for the treatment of non-Hodgkin's lymphoma, with a second anti-CD20 MAb labeled with ^{131}I , designated Bexxar,TM (Corixa, Seattle, WA) also being approved.^{1,2}

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Success in the treatment of solid tumors, however, has been limited and exemplifies the need to further refine radioimmunotherapeutic agents. One of the most important considerations for the designing of a radioimmunoconjugate (RIC) are the physical characteristics of the chosen radionuclide, i.e., the half-life and associated emissions as they directly relate to the therapeutic design and the disease being treated. The β -emitting radionuclides, such as ^{90}Y ($t_{1/2} = 2.67\text{d}$, $E_{\text{max}} = 2.3\text{ MeV}$) and ^{131}I ($t_{1/2} = 8.04\text{ d}$, $E_{\text{max}} = 0.81\text{ MeV}$), are readily available, and facile labeling procedures result in a RIC with high specific activity. One advantage of ^{90}Y over ^{131}I is that ^{90}Y deposits ~ 4.5 times more energy per mCi than ^{131}I . However, ^{131}I possesses an imageable γ -emission, a component that ^{90}Y lacks.³ The potential drawback of a radionuclide such as ^{90}Y is its mean path length in tissue of $\sim 2.5\text{ mm}$, with 90% of the energy deposited over a path length of 5.3 mm.⁴ With such a long path length, the majority of the energy from the decay of ^{90}Y would be deposited in tumors only if the diameter of the tumor mass were 1 cm or greater. A potential obstacle to consider is the hematologic toxicity in patients receiving ^{90}Y -labeled MAb.¹ There has been increasing interest and reports appearing in the literature on the development of α -emitting radionuclides for the treatment of smaller tumors/tumor burden, disseminated, and micrometastatic disease.^{5–12} Alpha-particle energy (4–9 MeV) is a discrete emission—not a spectrum, as with β -emissions—and is deposited over a small distance in tissue (40–100 μm), resulting in a high linear energy transfer. It is estimated that only 3–6 transversals of the nucleus are required to effect cell death, which correlates with an absorbed dose of 70–100 cGy.¹³ α -Particle radiation is cytotoxic at a dose rate as low as 1 cGy/hour¹⁴; thus, only a few molecules would be required to bind to a cell's surface to effect selective cell killing. The shorter penetration of the particle emission has also the potential of limiting toxicity to adjacent normal tissue. α -emitters currently under investigation for RIT applications are: ^{212}Bi ($t_{1/2} = 60.6\text{ minutes}$), ^{213}Bi ($t_{1/2} = 45.6\text{ minutes}$), ^{225}Ac ($t_{1/2} = 10\text{ days}$), and ^{211}At ($t_{1/2} = 7.2\text{ hours}$).^{5–11} Each of these radionuclides can be obtained in reasonable yields with a high degree of purity. ^{213}Bi is a decay product of ^{225}Ac and is readily obtainable from a $^{213}\text{Bi}/^{225}\text{Ac}$ generator.¹⁵ Use of an RIC labeled with ^{213}Bi has already been demonstrated in clinical trials.^{16,17} An added attraction to the

use of ^{213}Bi is its 440-keV α -emissions that are sufficient to perform real-time external imaging, which can be used in dosimetric calculations.¹⁷

The ^{213}Bi -labeled RIC's used in preclinical studies have included intact murine and humanized MAb, as well as fragments of MAb and a genetically engineered diabody and scFv.^{5–12} The slower pharmacokinetics of intact MAb molecules and their limited ability to penetrate solid tumors is thought to hinder their use for the delivery of short-lived radionuclides.¹⁸ Investigations with Fab' fragments have indicated that the ^{213}Bi -Fab' have been well tolerated in mice; however, pretreatment with D-lysine was required to minimize, but not completely eliminate, the higher renal uptake associated with this form of the MAb molecule.^{19–21} The scFvs have a more rapid plasma clearance as well as tumor penetration and might be an attractive moiety to deliver ^{213}Bi .^{22–24} However, this MAb form has a tendency toward a lowered tumor %ID/g and a significant renal uptake when labeled with radiometallonuclides.²⁵ Elimination of the extreme MW and size of the immunoprotein spectrum for the delivery of ^{213}Bi provides the hypothesis that the most promising form of MAb for this application would be a genetically engineered CH2 domain-deleted variant.

A chimeric domain-deleted variant of MAb CC49, a MAb that recognizes the tumor-associated glycoprotein TAG-72, has been shown in murine studies to have a rapid plasma clearance ($t_{1/2\alpha} = 3.4\text{ hours}$) and has demonstrated excellent tumor targeting radiolabeled with ^{177}Lu , without undue renal uptake.^{20,26} The humanized form of this variant, HuCC49 Δ CH2, has also been found to have favorable pharmacokinetics. At 24 hours following an i.v. injection, only 2% of the injected dose per g (%ID/g) of ^{131}I -HuCC49 Δ CH2 was in the blood compartment of athymic mice bearing s.c. LS-174T xenografts, while attaining a 11.1 %ID/g in the tumor.²⁷ ^{131}I -HuCC49 Δ CH2 is currently under evaluation in patients with metastatic colorectal carcinoma.²⁸ These properties provided the impetus for our hypothesis that HuCC49 Δ CH2 would be an appropriate candidate as a RIC with ^{213}Bi .

The study described herein evaluates the therapeutic potential of HuCC49 Δ CH2 labeled with ^{213}Bi , using CHX-A"-DTPA as the bifunctional chelating agent (BCA).^{17,29,30} The CHX-A"-DTPA has been shown to be appropriate for the complexation of Bi(III) radioisotopes and is the chelation component of the RIC evaluated in a

clinical trial with ^{213}Bi , as well as with other isotopes.⁶ This paper includes an assessment of escalating (μCi) doses of the ^{213}Bi -HuCC49 ΔCH2 , attempting to define a maximum tolerated dose. Based on the resulting data, additional experiments were performed to demonstrate the specificity of the therapeutic effect and to assess efficacy of dose fractionation.

MATERIALS AND METHODS

Mab HuCC49 ΔCH2

The development and characterization of MAb HuCC49 ΔCH2 have been described previously.^{27,31} MAb HuC49 ΔCH2 was purified from a bioreactor supernatant at the Monoclonal Antibody/Recombinant Protein Production Facility (Frederick, MD), which has also been detailed elsewhere.³²

Chelate Synthesis and MAb Conjugation

The synthesis, characterization, and purification of the bifunctional ligand, CHX-A"-DTPA, has been described previously.²⁹ The MAb HuCC49 ΔCH2 was conjugated with the CHX-A"-DTPA by established methods.¹⁵ The conjugation reaction was performed using a tenfold molar excess of ligand to MAb to insure that a final chelate-to-protein molar ratio of ~ 1 was obtained. In a previous study, the conjugation of HuCC49 ΔCH2 with a BCA had been found to severely affect the immunoreactivity of the MAb.³¹ Attempts to improve or retain the immunoreactivity of the MAb by lowering the molar ratio of ligand were futile. The final concentration of the HuCC49 ΔCH2 was quantitated by the method of Lowry.³³ The number of CHX-A" molecules linked to the MAb was determined using the spectrophotometric assay based on the titration of a Y(III)-Arsenazo(III) complex.³⁴ The immunoreactivity of HuCC49 ΔCH2 following conjugation with the BCA was evaluated utilizing a competition immunoassay that has been described elsewhere.³²

Radiolabeling of HuCC49 ΔCH2

Radiolabeling of CHX-A"-HuCC49 ΔCH2 (200 μg in 0.15 M of NH_4OAc buffer) with ^{111}In was performed by adding 2 mCi in 1–2 μL of ^{111}In -dium chloride (PerkinElmer, Boston, MA). The reaction was quenched by the addition of 5 μL of 0.05 M EDTA to scavenge any free radiometal.

Radioiodination of HuCC49 ΔCH2 with $\text{Na }^{125}\text{I}$ (PerkinElmer) was performed as previously described using the IODO-GEN, method (Pierce, Rockford, IL).³⁵ The radiolabeled products were purified using PD-10 desalting columns (Amersham Biosciences, Piscataway, NJ).

The labeling of HuCC49 ΔCH2 with ^{213}Bi was accomplished as follows: ^{213}Bi (7.8–8.4 mCi) was eluted from a ^{225}Ac (~ 12 mCi) generator (Oak Ridge National Laboratories, Oak Ridge, TN), as described previously.¹⁵ To the eluted ^{213}Bi (~ 1 mL), 100 μL (220 mg/mL) of ascorbic acid solution was added, and the pH of the mixture was adjusted to 4.5–5 with 5 M NH_4OAc . HuCC49 ΔCH2 -CHX-A" (300 μg) was then added to the ^{213}Bi solution. Following a 12-minute incubation at room temperature, 0.1 M EDTA (3 μL) was added to scavenge unreacted ^{213}Bi . The radiolabeled MAb was purified by size exclusion chromatography (SE-HPLC), using a TSK-3000 column (TosoHaas, Montgomeryville, PA), equilibrated in 100 mM sodium phosphate, with a pH of 7.4 at a flow rate of 1 mL/minute.

MAb HuCC49 ΔCH2 was labeled with ^{131}I using Iodo-Beads (Pierce).³⁶ Briefly, two beads were added to HuCC49 ΔCH2 (250 μg in 125 μL), followed by the addition of 5 mCi of $\text{Na }^{131}\text{I}$ (~ 5 μL ; PerkinElmer). Following a 5-minute incubation at ambient temperature, the ^{131}I -HuCC49 ΔCH2 was purified using PD-10 columns. A specific activity of 24 mCi/mg was obtained.

Immunoassays

The immunoreactivity of the HuCC49 ΔCH2 was evaluated in a competition immunoassay following conjugation with the CHX-A"-DTPA ligand. The assay was performed using the dissociation-enhanced, time-resolved fluorescence format and has been detailed elsewhere.³² Serial dilutions of the CHX-A"-DTPA-HuCC49 ΔCH2 were compared to the unmodified MAb, plotting the percent inhibition versus the competitor concentration. Human immunoglobulin served as the nonspecific antibody control.

The immunoreactivities of the radiolabeled preparations were assessed in a solid-phase radioimmunoassay, as detailed previously.²⁵ Bovine submaxillary mucin (BSM; 1 μg in PBS) was adsorbed to the wells of a microtiter plate. Following an overnight incubation at 4°C, the wells were blocked with 1% BSA in PBS just prior to use. Serial dilutions of radiolabeled HuCC49 ΔCH2 in 1% BSA in PBS were added to the wells in du-

plicate and incubated overnight at 4°C. The plates were then washed and the radioactivity measured in a γ -counter (Packard, Downers Grove, IL). The percent binding was calculated for each serial dilution. Wells coated with porcine submaxillary mucin (PSM) served as a negative control.

In Vivo Studies

Tumor targeting

The human colon adenocarcinoma cell lines, LS-174T and MIP, were obtained from the American Type Culture Collection (Manassas, VA) and maintained in supplemented Dulbecco's minimum essential medium, as previously described.³⁷ Four (4)–six-week-old female athymic (nu/nu) mice (Charles River Laboratories, Wilmington, MA) were injected subcutaneously (s.c.) in the flank with 1×10^6 cells in 0.2 mL of medium. Tumor-bearing mice were used in immunolocalization studies when the tumor xenografts maximal diameter measured 0.4–0.6 cm, approximately 10–14 days post-tumor-cell implantation. The tumor-bearing mice (5 per group) were injected intraperitoneally (i.p.) or intravenously (i.v.) with ^{111}In -CHX-A"-HuCC-49 Δ CH2 ($\sim 7.5 \mu\text{Ci}$). ^{125}I -HuCC49 Δ CH2 was coinjected to provide a comparison to published data.²⁷ The mice were then sacrificed by exsanguination at 1, 2, 4, and 24 hours. The blood, tumor, and major organs were collected, wet-weighted, counted in a γ -scintillation counter (Packard), and the percent injected dose per g (%ID/g) and standard deviation were calculated.

Therapy studies

RIT studies were performed when the LS-174T tumor xenografts measured ~ 80 – 100 mm^3 , ~ 12 days postimplantation. Doses of ^{213}Bi - or ^{131}I -labeled HuCC49 Δ CH2 were prepared in PBS and administered to the mice ($n = 5$ – 10) via i.p. injection. The mice were given 250, 500, 750, and 1000 μCi of ^{213}Bi -HuCC49 Δ CH2 in 1 mL of sterile PBS. The control group received PBS only. The average specific activity of the RIC was 11.4 mCi/mg. Mice were monitored for up to 154 days; tumors were measured 2–3 times weekly.

Mice were euthanized if they were found to be in distress, moribund, or cachectic. Mice were also euthanized when the tumor attained a diameter of 2 cm or developed an unhealing wound or if the mice experienced a 20% loss in weight. All animal protocols were approved by the

National Cancer Institute Animal Care and Use Committee.

Statistical analyses

Tumor volume was log-transformed ($\log(\text{volume} + 1)$) for analysis. The relationship between dose level and survival (i.e., time to sacrifice for large tumor volume) was determined using a Cox proportional hazards model, treating dose level as a continuous variable. A test of the dose–response relationship was performed with a likelihood-ratio test. The relationship between changes in tumor volume and dose level was assessed with the following two-stage procedure. First, because tumor growth was approximately linear on the log-scale, the slope was estimated for each mouse in the study. The relationship between dose level and mean slope were then tested using a linear regression. All reported p -values are two-sided.

RESULTS

Tumor Targeting and Biodistribution

Prior to RIT studies, a tumor-targeting experiment was performed to confirm that radiolabeled HuCC49 Δ CH2 would target a s.c. tumor when administered i.p. Athymic mice ($n = 5$) bearing s.c. LS-174T xenografts on the flank were injected with ^{111}In -CHX-A"-HuCC49 Δ CH2. The mice were euthanized at 1, 2, 4, and 24 hours, a time course relevant to the half-life of ^{213}Bi . Tumors and normal organs were collected, wet-weighted, and the radioactivity measured in a γ -counter. For a comparison to published studies, two additional groups of mice were injected i.v. via the tail vein and were sacrificed at 4 and 24 hours.²⁷ In addition, the mice were coinjected with ^{125}I -HuCC49 Δ CH2 again to serve as a comparison to previously published data.²⁷ Prior to injection, the radiolabeled preparations were tested for immunoreactivity in a solid-phase RIA. The specific binding of the radiolabeled preparations with TAG-72 positive BSM was 55% and 62% for the ^{111}In - and ^{125}I -labeled HuCC-49 Δ CH2, respectively. The nonspecific binding with the TAG-72 negative PSM was less than 1% for both preparations.

As shown in Table 1, i.p.-administered ^{111}In -HuCC49 Δ CH2 demonstrated excellent tumor targeting comparable to the ^{125}I -labeled HuCC-49 Δ CH2 at each of the time points, with a steady

Table 1. Biodistribution of Radiolabeled HuCC49ΔCH2 Administered to Athymic Mice Bearing LS-174T Human Colon Carcinoma Xenografts: Percent Injected Dose per g*

		<i>i.p. Injection</i>				<i>i.v. Injection</i>	
<i>Tissue</i>		<i>1 hr</i>	<i>2 hr</i>	<i>4 hr</i>	<i>24 hr</i>	<i>4 hr</i>	<i>24 hr</i>
Tumor	¹¹¹ In	6.6 ± 2.8	7.0 ± 1.2	12.7 ± 2.0	27.3 ± 5.5	31.8 ± 10.0	22.3 ± 4.8
	¹²⁵ I	6.2 ± 2.9	8.0 ± 2.9	13.6 ± 2.2	23.1 ± 4.4	32.8 ± 10.3	20.0 ± 4.4
Blood	¹¹¹ In	30.3 ± 5.6	17.0 ± 0.2	9.0 ± 4.4	0.5 ± 0.3	9.3 ± 1.1	0.5 ± 0.10
	¹²⁵ I	14.7 ± 1.9	12.3 ± 4.8	10.0 ± 1.8	0.5 ± 0.3	9.0 ± 1.1	0.6 ± 0.2
Liver	¹¹¹ In	4.2 ± 0.4	6.2 ± 0.5	9.3 ± 1.2	9.8 ± 1.1	11.8 ± 0.6	8.9 ± 3.4
	¹²⁵ I	4.6 ± 0.3	6.0 ± 1.1	6.6 ± 0.7	4.4 ± 0.1	6.3 ± 0.4	1.2 ± 0.5
Spleen	¹¹¹ In	8.3 ± 1.0	13.7 ± 1.5	19.4 ± 0.4	22.8 ± 4.0	21.0 ± 2.6	18.8 ± 8.2
	¹²⁵ I	9.5 ± 1.2	14.2 ± 3.4	13.8 ± 1.0	2.3 ± 0.5	14.1 ± 1.8	2.6 ± 1.0
Kidney	¹¹¹ In	7.6 ± 0.5	14.1 ± 0.3	22.8 ± 3.9	23.7 ± 2.3	28.0 ± 1.5	21.8 ± 8.3
	¹²⁵ I	8.7 ± 0.5	11.2 ± 1.9	8.4 ± 0.8	0.3 < 0.1	7.4 ± 0.4	0.4 ± 0.1
Lung	¹¹¹ In	4.9 ± 1.3	5.1 ± 0.2	4.7 ± 0.9	1.5 ± 0.1	5.1 ± 0.7	1.5 ± 0.5
	¹²⁵ I	5.7 ± 1.4	6.3 ± 2.1	6.1 ± 1.0	0.4 < 0.1	6.0 ± 0.8	0.6 ± 0.2

*Values are the percent injected dose per g (%ID/g) ± standard deviation.

increase from 6.6 ± 0.3 and $6.2 \pm 0.3\%$ ID/g at 1 hour to 27.3 ± 0.4 and 23.1 ± 0.1 by 24 hours for the ¹¹¹In- and ¹²⁵I-HuCC49ΔCH2, respectively. Additional mice were injected with ¹¹¹In- and ¹²⁵I-labeled HuCC49ΔCH2 in a separate experiment to evaluate the pharmacokinetics of the radioimmunoconjugate (Fig. 1). The ¹¹¹In-HuCC49ΔCH2 reached its maximum peak between 1 and 2 hours, achieving a %ID/mL of blood of 23.3 ± 3.0 that declined to 14.6 ± 2.3 by 6 hours. The ¹²⁵I-HuCC49ΔCH2 exhibited a similar pattern, differing only in the values at-

tained in the blood (i.e., a maximum %ID/mL of 27.0 ± 3.6).

Radioimmunotherapy

A RIT study was then performed to determine the maximum tolerated dose (MTD) of i.p.-administered ²¹³Bi-HuCC49ΔCH2 in groups of 5–10 mice bearing the LS-174T human colon carcinoma xenograft. The tumor xenografts grew rapidly (Fig. 2A), achieving an average volume of $545 \pm 152 \text{ mm}^3$ and $1261 \pm 226 \text{ mm}^3$ at 7 and 14 days, respectively, following RIT. One animal was euthanized by day 7, when the tumor became necrotic; the remainder of the animals were euthanized on day 15 post-RIT because of large tumor volumes. In the group of mice receiving 250 μCi of ²¹³Bi-HuCC49ΔCH2 (Fig. 2B), there was no apparent difference in the average tumor growth when compared to the PBS-injected group on day 7; however, there was a slight delay in the time required for the tumor to double in volume. The control group had doubled in volume by 2 days, while the 250 μCi group required 4 days to show an equivalent size increase. The average tumor volume was $77.2 \pm 32.7 \text{ mm}^3$, $288.4 \pm 150.3 \text{ mm}^3$, and $810.2 \pm 457.9 \text{ mm}^3$ on days 0, 7, and 14, post-RIT. Six (6) of 7 mice were sacrificed between 14 and 21 days postinjection because of tumor growth; 1 mouse in this group showed negligible tumor growth, with a tumor at 68.4 mm^3 on the day of treatment and reaching a volume of only 133.3 mm^3 by day 42.

Figure 1. Pharmacokinetics of ¹¹¹In-HuCC49ΔCH2. The pharmacokinetics of ¹¹¹In-HuCC49ΔCH2 (□) coinjected with ¹²⁵I-HuCC49ΔCH2 (■) following i.p. injection was evaluated in athymic mice bearing LS-174T tumor xenografts. The percent injected dose per mL of blood was calculated and plotted along with the standard deviation.

Figure 2. Radioimmunotherapy of athymic mice bearing s.c. LS-174T xenografts with ^{213}Bi -HuCC49 Δ CH2. Mice bearing 10–12 day s.c. tumor (averaging $86.6 \pm 43.9 \text{ mm}^3$) were treated with increasing doses of ^{213}Bi -HuCC49 Δ CH2: Panel: (A), PBS-treated; (B) 250 μCi ; (C) 500 μCi ; (D) 750 μCi ; and (E) 1000 μCi ^{213}Bi -HuCC49 Δ CH2. Each line represents the tumor growth in an individual mouse; measurements were taken 2–3 times per week. The day of treatment with ^{213}Bi -HuCC49 Δ CH2 is indicated with an arrow.

Delay of tumor growth was more evident in the mice that received 500 μCi of ^{213}Bi -HuCC49 Δ CH2 (Fig. 2C). In those tumors in which growth progressed, a doubling of tumor volume was attained 4 days following RIT. On the day of RIT, the average tumor volume was $94.3 \pm 29.5 \text{ mm}^3$ while on days 7 and 14 the average volumes were $252.5 \pm 152.3 \text{ mm}^3$ and $392.8 \pm 293.2 \text{ mm}^3$. Of the 8 mice that were followed, 2 mice demonstrated no tumor growth, 1 of which remained so until the study was terminated at 154 days; the other mouse was prematurely euthanized on day 34 (animal care facility personnel). Three other animals in this group demonstrated a significant delay in growth that did not appear to resume until between days 8 and 10, and thereafter, the growth was not as rapid versus control animals.

A greater reduction in tumor growth was observed in the group of mice that were injected with 750 μCi of ^{213}Bi -HuCC49 Δ CH2. The initial volume of the tumors were $79.2 \pm 18.2 \text{ mm}^3$. At day 7 and 14, the average tumor volumes obtained were $188.6 \pm 89.3 \text{ mm}^3$ and $325.4 \pm 227.8 \text{ mm}^3$. Again, in the mice in which the tumors appeared nonresponsive to RIT, tumors

doubled in volume between days 5 and 7. Of the remaining surviving animals, tumors resumed growth in 2 mice between days 10 and 15, while the tumors in the remaining 4 mice demonstrated no evidence of growth.

In the group of mice that received 1000 μCi of the ^{213}Bi -HuCC49 Δ CH2, tumors in 2 mice doubled in volume 6 days post-treatment. Of the remaining 8 mice, tumor growth was static until 17–20 days, after which tumor growth resumed in 4 of the mice. One mouse demonstrated a complete regression of tumor growth, and by day 35 no tumor growth was evident. Unfortunately, 3 mice in this group were also lost due to premature euthanasia (*vide infra*). For comparison to the other treatment groups, the average tumor volumes were $91.5 \pm 30.4 \text{ mm}^3$, $166.6 \pm 76.5 \text{ mm}^3$, and $195.9 \pm 96.8 \text{ mm}^3$ on days 0, 7, and 14, respectively.

Overall survival is presented in Figure 3A. No PBS-treated animals were alive at 30 days, whereas, 14%, 50%, 43%, and 33% of the mice receiving 250, 500, 750, and 1000 μCi , respectively, survived. By day 84, the remainder of the mice that had received 250 μCi of ^{213}Bi -HuCC49 Δ CH2 were euthanized because of ex-

Figure 3. Survival of tumor bearing mice following radioimmunotherapy with ^{213}Bi -HuCC49 ΔCH2 . Mice bearing 10–12 day s.c. tumors were treated with increasing doses of ^{213}Bi -HuCC49 ΔCH2 (**Panel A**). Kaplan-Meier curves are depicted as follows: (●), PBS-treated; (▼) 250 μCi ; (■) 500 μCi ; (◆) 750 μCi ; and (▲) 1000 μCi ^{213}Bi -HuCC49 ΔCH2 . Those mice who were alive after 84 days remained so until the experiment was terminated on day 154. The day of treatment with ^{213}Bi -HuCC49 ΔCH2 is indicated by the arrow. Boxplots of the estimated slopes for each dose level (**Panel B**). The light dotted line is the median, the upper portion of the box represents the third quartile, the lower portion is the first quartile, the brackets delineate 1.5 times the interquartile range, and the lines outside of the brackets represent outlying observations.

cessive tumor volume, as was 1 mouse in the 1000 μCi group. Those mice who were alive at this time remained so until the experiment was terminated on day 154. None of these remaining mice showed any evidence of tumor: 3 each in the 500 μCi and 750 μCi groups, and 1 in the 1000 μCi group. The median survival times were 16, 25, 84, 65 and 53 days for the PBS-treated, 250 μCi , 500 μCi , 750 μCi and 1000 μCi , respectively. Higher doses were problematic because of the availability and activity of $^{225}\text{Ac}/^{213}\text{Bi}$ generators. Statistical analyses of the tumor volume versus μCi dose level illustrated

in Figure 3B suggests that there is a strong dose response. The higher dosages resulted in smaller increases in the volume of the tumor xenografts ($p < 0.0001$).

Because another CH2 domain-deleted MAb was not available to serve as a control for evaluating nonspecific effects of a ^{213}Bi -labeled MAb, an experiment to demonstrate specificity of the RIT was designed, utilizing another human colon carcinoma xenograft (MIP), which does not express TAG-72, the cognate antigen of HuCC9 ΔCH2 . Athymic mice were implanted s.c. with either the LS-174T or MIP cell line and after 12–13 days, received either PBS or 1 mCi of ^{213}Bi -HuCC49 ΔCH2 by i.p. injection. As shown in Figure 4, in mice bearing the LS-174T xenografts that had received PBS (**Panel A**), the volume of the tumors increased dramatically. On the day of RIT, the average tumor size was $122.2 \pm 96.3 \text{ mm}^3$. Seven (7) days later, the average tumor volume was approximately 1100 mm^3 , a ninefold increase in volume. Because of large tumor volumes, 1 mouse was sacrificed on day 6, 1 on day 10, and 4 on day 13. In contrast, the group that received 1 mCi of ^{213}Bi -HuCC49 ΔCH2 (**Panel B**) showed an overall delay in tumor growth of 2 weeks following RIT. On the day of treatment with ^{213}Bi -HuCC49 ΔCH2 , the average tumor volume was $76.3 \pm 117 \text{ mm}^3$. On days 7, 14, and 21, the average tumor volumes were approximately 400, 700, and 1800 mm^3 . Excluding the three mice with LS-174T xenografts that appeared to be nonresponsive to the treatment, the average tumor volumes were 200, 500, and 1300 mm^3 . In comparing the PBS-treated group to the group treated with ^{213}Bi -HuCC49 ΔCH2 , there was a significant difference in the tumor growth ($p < 0.0001$). In fact, by day 13, 4 of the mice in the PBS-treated group were euthanized as a result of the large tumor volume. None of the mice demonstrated overt effects of radiation sickness (i.e., weight loss, diarrhea, etc.). In contrast, animal deaths resulting from radiation were observed in 5 of 9 (56%) mice bearing the TAG-72 negative MIP xenograft, and occurred on days 5, 7, and 10, following RIT (**Fig. 4D**). The mice experienced a rapid weight loss of $>20\%$, became cachectic, and developed petechiae. When the tumor growth of the treated mice was compared to the PBS-injected animals, little difference was observed. On day 0, the average volume of the PBS-treated tumors is $21.1 \pm 9.8 \text{ mm}^3$, and those treated with ^{213}Bi -HuCC49 ΔCH2 is $35.4 \pm 16.9 \text{ mm}^3$. Twenty-one

Figure 4. Specificity of radioimmunotherapy of ^{213}Bi -HuCC49 ΔCH2 . Athymic mice bearing the human colon carcinoma xenografts, the TAG-72 positive LS-174T (**Panels A and B**) or TAG-72 negative MIP (**Panels C and D**) were treated with phosphate-buffered saline (**Panels A and C**) or 1 mCi of ^{213}Bi -HuCC49 ΔCH2 (**Panels B and D**) to demonstrate the specificity of the therapeutic effect of ^{213}Bi -HuCC49 ΔCH2 on tumor growth. Treatment with ^{213}Bi -HuCC49 ΔCH2 is indicated by the arrow. The crosses indicate animal deaths.

(21) days following RIT, the approximate tumor volumes were 100 and 80 mm³, respectively. Twenty-eight (28) days were required for the PBS-treated tumors to attain a tumor volume of 200 mm³ and those treated with the ^{213}Bi -HuCC49 ΔCH2 did not reach the same volume until 31 days, a modest delay of 3 days in tumor growth. At 42 days following RIT, there were no significant differences between the 2 groups of mice in tumor volume. The group that was administered PBS had a tumor volume of 507.9 ± 290.4 mm³; the tumors of the group injected with the ^{213}Bi -HuCC49 ΔCH2 were 505.1 ± 96.4 mm³.

A study was then designed to assess the effectiveness of multiple doses versus a single dose of this RIC when 500 μCi was administered one, two, or three times at 2-week intervals. Consistent with the previous experiments, the LS-174T xenografts of the PBS-treated mice (Fig. 5) grew aggressively and attained an average volume of $2,216 \pm 742$ mm³ at 24 days following tumor

implantation, an increase of thirty-eight-fold. On day 12, when the first dose of ^{213}Bi -HuCC49 ΔCH2 was administered, the average tumor volume (all groups) was 58.8 ± 33.3 mm³. The average size of the xenografts 13 days after a single i.p. injection of ^{213}Bi -HuCC49 ΔCH2 was 978 ± 533 mm³. The xenografts attained a volume of 2073 ± 1213 mm³ by day 37, approximating the size of the 24-day PBS-treated xenografts. In those mice that received two 500 μCi doses, the average tumor volume was 1573 ± 834 mm³ on day 27 postinitiation of the RIT regimen, and it was not until approximately day 42 that the tumors attained a size that was 10% or more of the total body weight of the mouse. The third dose of ^{213}Bi -HuCC49 ΔCH2 (Fig. 5, Panel D) arrested tumor growth, which resumed ~ 10 days later. Overall, the administration of multiple doses, as compared to a single dose, appeared to increase animal survival by delaying tumor progression (Table 2). Of the PBS-treated mice, only

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Figure 5. Effect of a multiple-dose regimen of ^{213}Bi -HuCC49 ΔCH2 on the growth of LS-174T xenografts. The arrows indicate the days on which mice bearing LS-174T s.c. xenografts were given i.p. injections of ^{213}Bi -HuCC49 ΔCH2 (500 μCi per injection). Panels: (A), PBS-treated; (B), 500 $\mu\text{Ci} \times 1$; (C), 500 $\mu\text{Ci} \times 2$; (D), 500 $\mu\text{Ci} \times 3$.

20% remained alive 38 days after the tumor cells were implanted. On day 53, 13 days after the last dose of ^{213}Bi -HuCC49 ΔCH2 was administered, 3 remained that had received a single dose, 1 animal survived that had received two doses, while

the entire group of 8 that had been treated with three doses survived, and none of the PBS group remained ($p < 0.0001$).

To assess the effectiveness of RIT with an α -emitting radionuclide, ^{213}Bi was compared to

Table 2. Effect of Dose Fractionation of ^{213}Bi -HuCC49 ΔCH2 on the Survival of Athymic Mice Bearing s.c. Human Colon Carcinoma (LS-174T) Xenografts

Dose*	Total dose	# of Mice	Number of mice surviving (days post-tumor implantation)			
			12	23	38	53
PBS	0	10	10	9	2	0
500 $\mu\text{Ci} \times 1$	500 μCi	9	9	7	7	3
500 $\mu\text{Ci} \times 2$	1000 μCi	8	8	8	7	1
500 $\mu\text{Ci} \times 3$	1500 μCi	8	8	8	8	8

*The appropriate groups of mice were given i.p. injections of ^{213}Bi -HuCC49 ΔCH2 12, 27, and 40 days following tumor implantation. s.c., small-cell.

^{131}I , which has been used extensively in pre-clinical and clinical investigations.¹ Therefore, an experiment was conducted in which tumor bearing mice ($n = 10$) were injected with increasing doses (250–2000 μCi) of ^{131}I -HuCC49 ΔCH2 (Fig. 6). In this particular study, all PBS-injected animals were dead 22 days after treatment. Fifty percent (50%) of the animals treated with ^{131}I -HuCC49 ΔCH2 were alive at 22, 32, 46, 54, and 64 days for the 250, 500, 750, 1000, and 2000 μCi groups, respectively. On day 64, when the experiment was terminated, 58% of the group injected with 1500 μCi of ^{131}I -HuCC49 ΔCH2 were alive. Survival was found to be dose-dependent ($p = 0.0002$).

DISCUSSION

There have been a number of preclinical investigations, *in vitro* and *in vivo*, demonstrating the feasibility and potency of immunoconjugates labeled with α -particle emitting radionuclides (i.e., ^{211}At , ^{212}Bi , ^{213}Bi , and ^{212}Pb).^{8,10,11,37,38} This study represents the first report of a humanized domain-deleted MAb (CC49) variant form labeled with ^{213}Bi for the radioimmunotherapy of tumor xenografts. The i.p. administration of the RIC ^{213}Bi -labeled HuCC49 ΔCH2 for the RIT studies was chosen based on an anticipated clinical application of ^{213}Bi -labeled MAb in the treatment of disseminated intraperitoneal diseases, such as ovarian cancer. The injection route and s.c. tumor model employed herein was also chosen for this preliminary study to: (1) demonstrate the ability of this engineered protein to effectively target tumors in conjunction with (2) the actual targeting of a tumor that could be closely and accurately monitored, as the i.p. disease model does not lend itself well to physically measure tumor response. To justify this approach, an initial study was performed to demonstrate the tumor targeting of s.c. LS-174T xenografts with ^{111}In -HuCC49 ΔCH2 . As expected, the ^{111}In -HuCC49 ΔCH2 reasonably targeted the tumor xenograft and was sufficiently rapid to justify treating the xenograft with ^{213}Bi (Table 1). A separate experiment to assess the pharmacokinetics of HuCC49 ΔCH2 from the peritoneum (Fig. 1) into the circulatory system was also performed, which demonstrated efficient trafficking into the blood to justify targeting the xenograft with ^{213}Bi . The differences noted between these two sets of data originate from radical differences in blood sampling, cardiac puncture versus tail vein sampling in conjunction with the processing and expression of the data. The tumor and organ biodistribution, as well as the pharmacokinetics of the radioimmunoconjugate in both sets of experiments, paralleled that of coinjecting ^{125}I -HuCC49 ΔCH2 .

A study to determine the maximum tolerated dose of ^{213}Bi -labeled HuCC49 ΔCH2 was then conducted. Twelve (12) days after the s.c. implantation of LS-174T xenografts, mice were given i.p. injections of 250, 500, 750, and 1000 μCi of ^{213}Bi -HuCC49 ΔCH2 . While no significant inhibition of tumor growth was evident in the group of mice that received the 250 μCi dose, 1 mouse did show a regression of tumor growth, which subsequently remained static. At the three

Figure 6. Survival of tumor-bearing mice following radioimmunotherapy with ^{131}I -HuCC49 ΔCH2 . Mice bearing 10–12 day s.c. tumor were treated with increasing doses (**Panel A**) of ^{131}I -HuCC49 ΔCH2 ; (●), PBS-treated; (////) 250 μCi ; (▼) 500 μCi ; (■) 750 μCi ; (▲) 1000 μCi ; (●) 1500 μCi ; and (---) 2000 μCi of ^{131}I -HuCC49 ΔCH2 . The day of treatment with ^{131}I -HuCC49 ΔCH2 is indicated by the arrow. **Panel B:** Boxplots of the estimated slopes for each dose level. The light dotted line is the median, the upper region of the box represents the third quartile, the lower portion is the first quartile, the brackets delineate 1.5 times the interquartile range, and the lines outside of the brackets represent outlying observations.

higher doses of ^{213}Bi -HuCC49 Δ CH2, significant inhibition of tumor growth, compared to the PBS-treated group, was achieved. Overall, 500, or 750 μCi doses resulted in a prolonged survival of tumor-bearing mice. The average tumor volume at treatment in this experiment was $86.6 \pm 43.9 \text{ mm}^3$. Studies with ^{212}Bi -labeled anti-Tac found the radioimmunoconjugate to be ineffective in the treatment of larger s.c. lymphoma xenografts.⁴⁰ A greater tumor response to RIT with the ^{213}Bi -HuCC49 Δ CH2 might have been obtained if mice with a smaller tumor burden were treated.

Interestingly, the initial objective of this experiment was not achieved because a MTD was not established, even at a dose of 1 mCi. Nikula et al. determined the MTD of ^{213}Bi -HuM195, an anti-CD33 MAb, to be 1.46 mCi when given by i.p. injection.⁴¹ However, this study was performed in normal, immunocompetent Balb/c mice with an intact MAb molecule. Athymic mice may not prove to be as robust as the Balb/c strain; however, higher levels of radiation may yet be administered when the rapid pharmacokinetics of the HuCC49 Δ CH2 is taken into consideration. Determination of the MTD will require another study with greater doses, as no overt toxicity was observed in mice at the highest doses used in this study.

The effectiveness of ^{213}Bi -HuCC49 Δ CH2 in inhibiting or reducing tumor burden was found to be greater, as compared to ^{131}I -HuCC49 Δ CH2. On day 64 (when the ^{131}I RIT study was terminated), therapy with the ^{213}Bi had greater efficacy at the 250 and 500 μCi doses, and 44%, 50%, 43%, and 57% of the mice were alive after receiving 250, 500, 750 and 1000 μCi , respectively, of ^{213}Bi -HuCC49 Δ CH2. In contrast, 10%, 24%, 50%, and 41% of the mice were alive after receiving the same doses of ^{131}I -HuCC49 Δ CH2.

Specificity of tumor-growth inhibition was demonstrated by comparing TAG-72 positive (LS-174T) to TAG-72 negative (MIP) human colon carcinoma xenografts. In the group of mice bearing the LS-174T xenografts, there was a significant ($p < 0.0001$) inhibition of tumor growth in those mice that received ^{213}Bi -HuCC49 Δ CH2 versus PBS. Conversely, the mice bearing MIP xenografts did not exhibit any differences in tumor growth between the two groups. Furthermore, 5 of 9 mice bearing MIP tumors treated with ^{213}Bi -HuCC49 Δ CH2 died 6–13 days following treatment.

The dose escalation study demonstrated therapeutic efficacy of ^{213}Bi -HuCC49 Δ CH2 in the de-

laying of tumor growth and/or in tumor regression. Previous RIT studies with MAb, such as ^{131}I -COL-1 or ^{177}Lu -CC49, using the LS-174T mouse model, have illustrated that a regimen of multiple doses of a RIC was more effective than a single treatment.^{42,43} Therefore, the efficacy of a multiple-dose regimen of ^{213}Bi -HuCC49 Δ CH2 was evaluated. The administration of multiple doses did appear to improve the survival of the tumor-bearing mice, and a delay in tumor growth was evident with the administration of each additional dose. However, the “cure” rate was not increased because only 1 mouse in each group showed evidence of tumor regression. This lack of cure rate may stem from: (1) a low tumor %ID/g of the ^{213}Bi -HuCC49 Δ CH2 at early time points; (2) the larger tumor burden present with each subsequent administration; and (3) not all cells in the tumor expressed the TAG-72 antigen. The LS-174T is a tumor that grows aggressively, and a regimen in which doses are administered 2 weeks apart may allow sufficient time for the resumption of tumor growth. As shown in Figure 5, Panel D, the third 500 μCi dose of ^{213}Bi -HuCC49 Δ CH2 did inhibit tumor growth; but tumor growth resumed after a delay, apparently at the same rate. As with earlier studies evaluating regimens of fractionated dosing, ^{213}Bi -HuCC49 Δ CH2 should most likely be administered at 1-week intervals, or at the minimum elapsed time that can be tolerated by mice in the appropriate model system.^{42,43}

It is remarkable that, in this study, a significant delay in the growth of an aggressive s.c. solid tumor was observed following treatment with a short-lived radionuclide. There remains a need for a viable explanation for there to be any significant therapy as achieved with α -particle RIT using such a solid tumor model system. Simple conventional thought does not immediately support this possibility because of the half-life of ^{213}Bi in conjunction with a probable lack of penetration by the delivery vector into the tumor to achieve a homogeneous distribution and, hence, the delivery of high LET radiation to all of the targeted cells. Reconciliation of the observed therapy herein requires consideration of other factors involved in tumor targeting to explain, at least in part, these results. One possible explanation of these observations lies within the pattern of tumor regrowth and delayed regrowth. The pattern of recurrence is consistent with the efficient killing of cells on the perimeter of the tumor and, also, the adjacent vasculature supplying

the tumor, where the RIC has been transported to the tumor cells and is bound. The targeting of vasculature, be it tumor associated or the targeting of normal vasculature adjacent to tumor cells,⁴⁴ have both been proposed as excellent targets for α -particle RIT.^{9,45} The results of this effect, in conjunction with a significant treatment of the entire perimeter, could be responsible for the eradication of tumors, if the encapsulation was both efficient and complete. The results of this action being incomplete would very clearly explain tumor responses, coupled with the delayed resumption of tumor growth being the result of cells that escaped RIT, or the incomplete closure of vasculature that then permits an influx of nutrients and/or a breakout of the tumor. While the evidence for this mechanism is far from complete, this also provides a viable explanation and an impetus to not only investigate higher doses but also to deliberately target the tumor vasculature, combined with tumor targeting.^{43,45} Yet another factor contributing to the eradication of tumor cells in our model could be a “bystander effect,” when irradiated cells send biological signals to nonirradiated cells, which results in cell death.⁴⁶

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