

# Development of a two-antibody model for the evaluation of copper-64 radioimmunotherapy

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## Abstract

Copper-64 emits  $\beta^+$  and  $\beta^-$  particles suitable for positron emission tomography and radioimmunotherapy (RIT) of cancer. Copper-64-labelled antibodies have caused complete responses in laboratory animal RIT studies at far lower radiation doses than traditionally prescribed. The intracellular localization of copper radioisotopes may lead to cytotoxic effects by mechanisms beyond ionizing radiation damage. The purpose of this research was to develop a model using both internalizing and non-internalizing antibodies for direct comparison in future RIT studies using the same animal model of cancer. The monoclonal antibodies, cBR96 and cT84.66, were conjugated with *N*-hydroxysulfosuccinimidyl DOTA. All conjugates retained high immunoreactivity and labelled efficiently with <sup>64</sup>Cu with high specific activity and radiochemical purity. Twenty-four hour biodistributions determined in LS174T tumour-bearing nude mice demonstrated low organ and high tumour uptakes for both monoclonal antibodies. This model constitutes a promising system for elucidating whether internalization of <sup>64</sup>Cu is responsible for an enhanced tumour cytotoxicity *in vivo*.

## Keywords

cancer, carcinoembryonic antigen, copper-64, Lewis<sup>Y</sup>, radioimmunotherapy

## Introduction

Radiolabelled monoclonal antibodies have shown considerable promise for tumour imaging and therapy. However, because of their slow blood clearance, they generally do not localize to solid tumours in sufficient quantities to provide consistent therapeutic efficacy without significant toxicity. It has been estimated that an absorbed dose of 54–80 Gy (5400–8000 rad) must be delivered to a tumour to effect a complete response (Hall,

2000). However, because of their extremely slow clearance properties, monoclonal antibodies labelled with conventional radionuclides usually cannot deliver this dose without unacceptable radiation toxicity to bone marrow, in the range of 1.5–2.0 Gy (150–200 rad) (Goldenberg, 2003). However, recent research with antibodies and other radiopharmaceuticals labelled with copper radioisotopes has shown that complete and durable

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regressions can be achieved in extremely aggressive rodent models of cancer at doses that are a small fraction of those prescribed for traditional therapeutic radionuclides, such as  $^{131}\text{I}$  and  $^{90}\text{Y}$ . The properties of copper radiopharmaceuticals offer the promise of more effective tumour therapy with substantially lower systemic toxicity.

Copper-64 ( $T_{1/2} = 12.7\text{ h}$ ) is an intermediate-lived radionuclide that decays by both  $\beta^+$  and  $\beta^-$  emission, making it suitable for labelling monoclonal antibodies, peptides and small molecules for both positron emission tomography (PET) imaging and targeted radiotherapy of cancer. The decay characteristics of  $^{64}\text{Cu}$ , as well as those of  $^{131}\text{I}$  and  $^{90}\text{Y}$ , are listed in Table 1. Listed are the  $\beta^+$ ,  $\beta^-$  and  $\gamma$  emissions that have utility in imaging and therapy. The 364-keV  $\gamma$  from  $^{131}\text{I}$  delivers a significant dose of radiation to the patient's body, but its energy and abundance are not optimal for high-resolution imaging, especially during the course of radioimmunotherapy (RIT). The  $\beta^+$  and  $\beta^-$  emissions of  $^{64}\text{Cu}$  travel only 2 mm in tissue, giving high resolution for PET imaging and exposing minimal normal tissue surrounding tumours to radiation damage. In contrast, the 2.27-MeV  $\beta^-$  of  $^{90}\text{Y}$  travels up to 11 mm in soft tissue, delivering a significant radiation doses to normal tissues, often resulting in dose-limiting toxicity. In studies comparing  $^{64}\text{Cu}$  with other radionuclides,  $^{64}\text{Cu}$ -labelled radiotherapeutic agents have demonstrated superior tumour cytotoxicity at far lower absorbed radiation doses.

In survival studies of Golden Syrian hamsters bearing 200-mg GW39 colorectal cancer xenografts

in the thigh musculature, animals treated with  $^{64}\text{Cu}$ -labelled monoclonal antibody 1A3 had an 82% 7-month survival and were microscopically free of disease at necropsy with a calculated tumour-absorbed dose of only 5.86 Gy (586 rad) (Connett *et al.*, 1996). This tumour response stands in stark contrast to results obtained with  $^{131}\text{I}$ - and  $^{90}\text{Y}$ -labelled monoclonal antibodies. Iodine-131-labelled anti-carcinoembryonic antigen (anti-CEA) monoclonal antibody NP-4 in GW39-bearing hamsters yielded a 55% complete remission rate at tumour-absorbed doses ranging from 24 to 72 Gy (2400 to 7200 rad) (Sharkey *et al.*, 1987; Blumenthal *et al.*, 1989a). However, the study in which  $^{131}\text{I}$ -labelled NP-4 effected responses at a tumour-absorbed dose of 24 Gy was carried out in hamsters bearing small tumours (<200 mg) in the cheek pouch. It is well established that smaller GW39 tumours implanted in this location, which has greater vascular volume and permeability than the thigh musculature, show significantly higher radio-labelled antibody targeting and delivery and thus, better tumour responses (Blumenthal *et al.*, 1989b). Nude mice bearing subcutaneous, 300-mg GW39 xenografts showed only tumour growth inhibition following treatment with  $^{90}\text{Y}$ -labelled NP-4 (Sharkey *et al.*, 1988), after receiving a tumour-absorbed dose of 16.03 Gy (1603 rad). Compared to hamsters, mice exhibit slower blood clearance and higher tumour uptake of radiolabelled monoclonal antibodies, often resulting in a better anti-tumour response (Blumenthal *et al.*, 1989b). Taken together, these studies suggest that  $^{64}\text{Cu}$ -labelled 1A3 exerted considerably greater therapeutic efficacy against

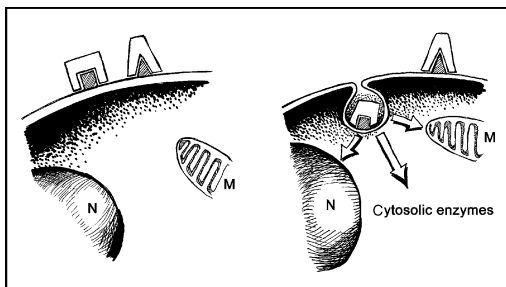
**Table 1.** Decay characteristics, energy of particle emissions and range of  $\beta^-$  particles in tissue of commonly used radionuclides for tumour therapy

Radionuclide	$T_{1/2}$ (h)	$\beta$ particle energy (MeV) and abundance (%)	Electron capture energy (MeV) and abundance (%)	$\gamma$ energy (keV) and abundance (%)	Maximum $\beta^-$ range in tissue (mm)
$^{64}\text{Cu}$	12.7	$\beta^+$ 0.655 (17.4%) $\beta^-$ 0.573 (39.0%)	0.33 (0.6%) 1.68 (40.5%)	1350 (0.6%)	2
$^{131}\text{I}$	192	$\beta^-$ 0.25 (2.8%) 0.33 (9.3%) 0.606 (87.2%) 0.806 (0.6%)	–	80 (2.6%) 284 (5.4%) 364 (82%) 637 (6.8%) 723 (1.6%)	3
$^{90}\text{Y}$	64.0	$\beta^-$ 2.27 (100%)	–	–	11

Percentages may total greater than 100 as some decay events leave the daughter nuclide at an elevated energy state resulting in a second emission of a  $\gamma$  photon.

GW39 xenografts, under less favourable conditions, at substantially lower tumour-absorbed doses than  $^{131}\text{I}$ - and  $^{90}\text{Y}$ -labelled monoclonal antibodies.

The efficacy of  $^{64}\text{Cu}$ -targeted radiotherapy has also been demonstrated in Lewis rats bearing CA20948 pancreatic tumours (Lewis *et al.*, 1999). Rats were treated with the somatostatin analogue tyrosine-3-octreotate or  $^{64}\text{Cu}$ -labelled tyrosine-3-octreotate in three fractionated 20-mCi doses at 48-h intervals. Although unlabelled somatostatin analogues have growth inhibitory properties and are used clinically for treatment of somatostatin receptor-positive tumours, animals treated with the unlabelled peptide demonstrated unrestricted tumour growth. Rats receiving  $^{64}\text{Cu}$ -labelled tyrosine-3-octreotate showed complete tumour regressions for 10 days, beginning 14 days after implantation. The cumulative tumour-absorbed dose was 13.03 Gy (1303 rad). Although the tumours eventually returned, recurrent tumours showed reduced growth rates, and the mean survival of  $^{64}\text{Cu}$ -treated rats was approximately twice that of animals receiving unlabelled tyrosine-3-octreotate. Achieving complete responses in aggressive rodent models of cancer with such low doses of  $^{64}\text{Cu}$  radiation represents a remarkable accomplishment. Yet, both of these  $^{64}\text{Cu}$  radiopharmaceuticals are rapidly internalized upon antigen or receptor binding, resulting in delivery of the radionuclide directly into potentially critical intracellular compartments. Figure 1 shows a graphic representation of the events following binding of an internalizing and a non-internalizing radiolabelled antibody.



**Figure 1.** Schematic representation of antibody fate after antigen binding. Non-internalizing antibodies (triangle) remain on the cell surface after binding. Internalizing antibodies (square) undergo antigen- or receptor-mediated endocytosis. After internalization, the  $^{64}\text{Cu}$  may be distributed to the nucleus (N), mitochondria (M) or cytosolic enzymes.

These observations suggest that mechanisms in addition to classical radiation damage may be responsible for the enhanced cytotoxicity of  $^{64}\text{Cu}$ . To this end, metabolism and subcellular fractionation studies have been performed. *In vivo* studies of  $^{64}\text{Cu}$ -labelled somatostatin analogues in rats have demonstrated that  $^{64}\text{Cu}$  was incorporated as a cofactor into hepatic superoxide dismutase (Bass *et al.*, 2000). Implicit in this finding is that the copper dissociated from the radiopharmaceutical inside cells and was capable of transchelating to biomolecules. *In vitro*, substantial accumulation of  $^{64}\text{Cu}$  has been found in the nucleus and the mitochondria of AR42J rat pancreatic carcinoma cells (Wang *et al.*, 2003). The results of these studies are provocative, raising the possibility that binding to critical nuclear structures, such as chromatin or nuclear matrix proteins, may cause lethal damage by mechanisms in addition to ionization. Furthermore,  $^{64}\text{Cu}$  accumulation may cause mitochondrial DNA damage, potentially resulting in respiratory cell death.

What has not been demonstrated conclusively is that the internalization of  $^{64}\text{Cu}$  is required in order to kill tumours at lower radiation doses. The purpose of this research was to construct an *in vivo* model by which to compare directly internalizing and non-internalizing  $^{64}\text{Cu}$ -labelled monoclonal antibodies in the same animal model of cancer. The LS174T colorectal carcinoma cell line is tumorigenic in nude mice and expresses both the tumour-associated Lewis<sup>x</sup> ( $\text{Le}^x$ ) carbohydrate ceramide variant (Trail *et al.*, 1999) and CEA (Tom *et al.*, 1977) on the cell membrane. The monoclonal antibody cBR96 recognizes  $\text{Le}^x$  and is rapidly internalized upon binding (Hellström *et al.*, 1990). The monoclonal antibody cT84.66 recognizes CEA and is not internalized upon binding (Neumaier *et al.*, 1990). Both antibodies are minimally cross-reactive in normal murine tissues (Hellström *et al.*, 1990; Neumaier *et al.*, 1990). This two-antibody model system was designed to allow direct comparison of internalizing versus non-internalizing antibodies in RIT studies using the same animal model, to test the hypothesis that internalization is necessary for an enhanced tumour cell killing by  $^{64}\text{Cu}$ .

## Materials and methods

### Antibodies

The human/murine chimeric antibody cBR96, isotype IgG<sub>3</sub>, was provided by Seattle Genetics (Bothell, WA, USA). The DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) conjugate of the human/murine chimeric monoclonal antibody cT84.66 (Lewis *et al.*, 2001a), isotype IgG<sub>1</sub>, was a gift from the City of Hope National Medical Center (Duarte, CA, USA).

### DOTA Conjugation

An aliquot of 15 mg of cBR96 was conjugated with the bifunctional chelating agent *N*-hydroxysulfosuccinimidyl DOTA (DOTA-OSSu) in 5:1, 10:1 and 20:1 molar ratios of DOTA-OSSu: monoclonal antibody. Conjugation was performed using the active ester method previously described by Lewis *et al.* (Lewis *et al.*, 1994; Lewis *et al.*, 2001a). The monoclonal antibody cT84.66 was conjugated at a DOTA-OSSu: monoclonal antibody 20:1 molar ratio, using the same method.

### Conjugate analysis

The purity of the conjugates was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion high-performance liquid chromatography (SE-HPLC). Immunoreactivity was determined for all cBR96 conjugates by an enzyme-linked immunosorbent assay (ELISA), using a purified Le<sup>y</sup>-human serum albumin conjugate (Seattle Genetics) and unconjugated cBR96 as the positive control. The immunoreactivity of DOTA-cT84.66 was previously determined by incubation with purified CEA, followed by SE-HPLC analysis (Lewis *et al.*, 2001a). The number of functional chelates per antibody was determined for each conjugate using a modification of the method previously published by Meares *et al.* (Meares *et al.*, 1984). DOTA-cBR96 and DOTA-cT84.66 were incubated with a 10-fold molar excess of CuCl<sub>2</sub> (99.995%, Alfa Aesar, Ward Hill, MA, USA), trace labelled with <sup>64</sup>CuCl<sub>2</sub>, in 0.1 M ammonium citrate, pH 5.5, for 1 h at 43 °C.

The chelator diethylenetriaminepentaacetic acid (DTPA) was then added to a final concentration of 1 mM to remove unbound copper. The reaction mixtures were incubated for 15 min at room temperature and analysed by SE-HPLC. SE-HPLC was performed on a Waters Delta 600 (Waters, Milford, MA, USA) chromatograph equipped with a manual Rheodyne injector, a 2487 dual wavelength UV detector, a Packard 500TR Flow Scintillation Analyser (Packard, Downers Grove, IL, USA) with a GAMMA-C flow cell for <sup>64</sup>Cu, a Waters busSAT/IN analog-digital interface, and the Millennium 32 software package. A Phenomenex BioSep-SEC-S 3000 (Phenomenex, Torrance, CA, USA) column (7.8 × 300 mm, 5 μm, 290 Å), an isocratic mobile phase of 100 mM NaH<sub>2</sub>PO<sub>4</sub>/0.05% NaN<sub>3</sub>, pH 6.8 and a flow rate of 1.0 mL min<sup>-1</sup> were used.

### <sup>64</sup>Cu labeling

Copper-64 was produced on a biomedical cyclotron at Washington University School of Medicine by previously published methods (McCarthy *et al.*, 1997). All conjugates were labelled with <sup>64</sup>Cu at increasing specific activities from 1 μCi μg<sup>-1</sup> to 20 μCi μg<sup>-1</sup> in 0.1 M ammonium citrate, pH 5.5, for 1 h at 43 °C (Lewis *et al.*, 2001a), after which DTPA was added to a final concentration of 1 mM. After 15 min at room temperature, the labelled monoclonal antibodies were purified and exchanged into phosphate-buffered saline by gel-filtration spin column chromatography, using Bio-Spin 6 columns (Bio-Rad, Hercules, CA, USA) (Lewis *et al.*, 2001b). Specific activity of labelling was determined prior to purification, and radiochemical purity was determined after purification by SE-HPLC.

### Serum stability studies

Serum stability analysis was performed by adding 1 mCi of <sup>64</sup>Cu-labelled DOTA-cBR96 or DOTA-cT84.66 to a screw-cap polypropylene tube containing 1 mL of mouse serum, clarified by centrifugation at 16 110 g for 15 min and stabilized with 10 μL of 10% NaN<sub>3</sub>. Serum samples were analysed by SE-HPLC after incubation at 37 °C for 15 min, 3 h, 6 h, 24 h and 48 h.

## Animal model

All animal experiments were conducted in compliance with the guidelines established by the Animal Care and Use Committee of the University of Missouri-Columbia Animal Care Quality Assurance Office. Outbred female nu/nu mice (4–6 weeks of age) were obtained from Harlan Bioproducts (Indianapolis, IN, USA). They were implanted subcutaneously with  $1 \times 10^6$  LS174T (The American Type Culture Collection, Manassas, VA, USA) colorectal tumour cells in the right hind flank. After 14 days, tumours grew to a mean weight of 223 mg.

## Biodistribution studies

Samples for injection were prepared by labelling all conjugates with  $^{64}\text{Cu}$ , as described above, at a ratio of  $10 \mu\text{Ci} \mu\text{g}^{-1}$ . After purification, the radiolabelled monoclonal antibodies were diluted to a concentration of  $0.1 \mu\text{Ci} \mu\text{L}^{-1}$  with sterile normal saline for injection. For each conjugate, doses of  $10 \mu\text{Ci}$  were given intravenously (i.v.) via the tail vein to a group of five tumour-bearing mice. Mice were killed exactly 24 h after injection and the following tissues were collected: blood, liver, spleen, kidneys, heart, lung, tumour, muscle, bone, small intestine, large intestine and stomach. Each tissue was weighed, placed in a vial and counted using a Wallac 1480 Wizard 3'' automated gamma counter (PerkinElmer Life Sciences, Gaithersburg, MD, USA). Tissues from each mouse were counted with an empty vial and a standard of 1% of the injected dose, such that background- and decay-corrected uptakes were calculated as the percent injected dose per gram of tissue (% ID/g).

## Statistical analysis

Mean tissue uptakes were compared among groups, by one-way analysis of variance (ANOVA) using statistical software (SPSS, Chicago, IL, USA). Where significant differences were observed, pairwise multiple comparisons were performed using the Tukey procedure. Differences at the 95% confidence level ( $P < 0.05$ ) were considered significant.

## Results

### Conjugates

SDS-PAGE and SE-HPLC confirmed all three cBR96 conjugates to be homogeneous, with minimal evidence of cross-linking or aggregation ( $<1\%$ ). Relative immunoreactivity for each DOTA-cBR96 conjugate was as follows: 5:1,  $99.3 \pm 11.3\%$ ; 10:1,  $114.6 \pm 8.9\%$ ; 20:1,  $86.1 \pm 16.4\%$ . The 10:1 DOTA-cBR96 conjugate showed the highest immunoreactivity,  $114.6\%$  compared to unconjugated cBR96. Although this value may seem artificially high, it was extremely reproducible and consistent with the observed inter-assay variability of approximately 15% for the ELISA. Moreover, the immunoreactivity of  $86.1\%$  determined for the 20:1 cBR96 conjugate was also within inter-assay variability. Immunoreactivity of the 20:1 DOTA-cT84.66 conjugate has previously been determined to be 100% by reaction with purified antigen and SE-HPLC (Lewis *et al.*, 2001a).

The number of functional chelates per antibody molecule increased proportionally for each DOTA-OSSu : monoclonal antibody conjugation ratio. The average numbers of chelates per antibody for the cBR96 conjugates were: 0.54 at a conjugation ratio of 5:1, 1.26 at a 10:1 ratio and 3.43 at a 20:1 ratio. This translated to a conjugation reaction efficiency of approximately 13–17%. The DOTA-cT84.66 conjugation ratio of 20:1 yielded 3.6 functional  $^{64}\text{Cu}$  chelates per antibody, a value consistent with that previously reported using the  $\gamma$ -emitting radiometal  $^{111}\text{In}$  (Lewis *et al.*, 2001a). At a DOTA-OSSu : monoclonal antibody ratio of 20:1, both cBR96 and cT84.66 had very similar degrees of chelate modification.

### Radiolabelling

All DOTA-cBR96 conjugates labelled efficiently and reproducibly with  $^{64}\text{Cu}$  to specific activities as high as  $15 \mu\text{Ci} \mu\text{g}^{-1}$ . Radiochemical purity of each conjugate was uniformly greater than 99.4% after purification. For both  $^{64}\text{Cu}$ -DOTA-cBR96 and  $^{64}\text{Cu}$ -DOTA-cT84.66, the serum stability was demonstrated to be 100% over 48 h under physiologic conditions.

## Biodistributions

The *in vivo* distributions of the four conjugates are presented in Table 2. At 24-h post-injection, the tissue containing the most radioactivity for each of the cBR96 conjugates was the tumour. The second highest concentration of  $^{64}\text{Cu}$  was found in the blood for all DOTA-cBR96 conjugates. All  $^{64}\text{Cu}$ -DOTA-cBR96 conjugates showed low liver, spleen, kidney and bone uptakes. Tumor delivery was extremely high, at values ranging 43.3–48.8% ID/g. There were no statistically significant differences in the tumour uptakes of the three DOTA-cBR96 conjugates. The DOTA-cT84.66 biodistribution was similar to the cBR96 conjugates, with the exceptions of increased uptakes in liver (33.8% ID/g), spleen (17.1% ID/g) and tumour (74.0% ID/g). These values were significantly different from the values for the cBR96 conjugates (liver and spleen,  $P < 0.001$ ; tumour,  $P = 0.007$ ). Tumor-to-normal tissues ratios of  $^{64}\text{Cu}$ -DOTA-cBR96 and  $^{64}\text{Cu}$ -DOTA-cT84.66 uptakes are shown in Fig. 2. Tumor-to-blood, tumour-to-liver, tumour-to-spleen, tumour-to-kidney and tumour-to-bone ratios were calculated. There were no systematic statistically significant differences among these conjugates.

## Discussion

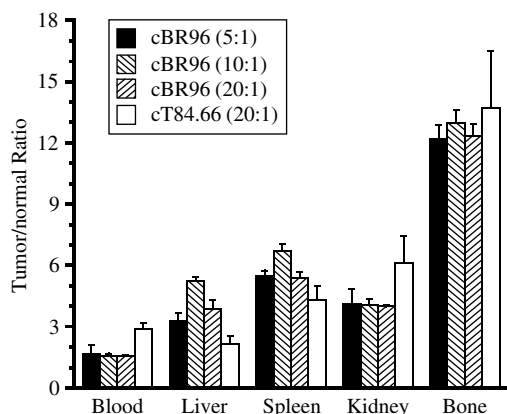
The DOTA-antibody conjugation method used for these experiments was robust and highly reproducible. The active ester of DOTA reacts

predominantly with lysine residues on the monoclonal antibody, of which there are 24 in the two antigen-combining sites of cBR96 (Francisco, J., personal communication). Over-conjugation can result in conformational changes in the antigen-combining site and loss of immunoreactivity. Multiple activation of DOTA can also cause dimerization or aggregation of monoclonal antibodies, resulting in loss of function. SDS-PAGE and SE-HPLC analysis of the cBR96 conjugates demonstrated that the products were homogeneous, with minimal (<1%) cross-linking. The ELISA results demonstrated high immunoreactivity for all of the conjugates. For the purpose of optimizing specific delivery of radioactivity to tumours, maximal immunoreactivity, is desirable. As the 10:1 DOTA-cBR96 conjugate consistently displayed the highest immunoreactivity as well as reproducibly high specific activity radiolabelling, this conjugation ratio was selected as optimal for future studies.

The labelling of all conjugates with  $^{64}\text{Cu}$  was very efficient at high specific activities and yielded reproducibly pure products. Specific activity correlates with the amount of radioactivity that can be delivered to tumours per mass unit of monoclonal antibody injected. The specific activities obtained for  $^{64}\text{Cu}$ -labelled DOTA-cBR96 and DOTA-cT84.66, 10–15  $\mu\text{Ci } \mu\text{g}^{-1}$ , are suitable for clinical PET imaging and RIT applications. The radiochemical purity of the  $^{64}\text{Cu}$ -labelled monoclonal antibodies assures that all of the radioactivity administered is in the desired form of the

**Table 2.** Biodistributions ( $n = 5$ ), at 24-h post-injection, of  $^{64}\text{Cu}$ -labelled DOTA-cBR96 and DOTA-cT84.66 conjugates, expressed in percent injected dose per gram of tissue (% ID/g)  $\pm$  standard error of the mean

Tissue	cBR96 (5 : 1)	cBR96 (10 : 1)	cBR96 (20 : 1)	cT84.66 (20 : 1)
Blood	26.0 $\pm$ 1.53	29.4 $\pm$ 2.03	30.7 $\pm$ 1.45	25.5 $\pm$ 3.00
Lung	11.0 $\pm$ 0.66	11.9 $\pm$ 1.15	12.1 $\pm$ 0.48	13.0 $\pm$ 0.91
Liver	13.1 $\pm$ 2.09	8.76 $\pm$ 0.44	12.6 $\pm$ 1.55	33.8 $\pm$ 2.82
Spleen	7.90 $\pm$ 1.11	6.88 $\pm$ 0.37	8.99 $\pm$ 0.48	17.1 $\pm$ 1.19
Kidney	10.5 $\pm$ 0.63	11.3 $\pm$ 0.52	12.2 $\pm$ 0.28	12.1 $\pm$ 1.38
Muscle	2.38 $\pm$ 0.20	2.25 $\pm$ 0.16	2.73 $\pm$ 0.36	2.56 $\pm$ 0.33
Heart	7.88 $\pm$ 0.60	8.19 $\pm$ 0.68	9.28 $\pm$ 0.49	8.51 $\pm$ 0.76
Bone	3.55 $\pm$ 0.07	3.56 $\pm$ 0.31	3.95 $\pm$ 0.42	5.40 $\pm$ 0.65
Stomach	2.31 $\pm$ 1.73	3.33 $\pm$ 0.43	3.23 $\pm$ 0.34	3.03 $\pm$ 1.25
Small intestine	11.2 $\pm$ 1.73	7.35 $\pm$ 0.43	7.86 $\pm$ 0.34	5.26 $\pm$ 1.25
Large intestine	7.94 $\pm$ 1.10	5.29 $\pm$ 0.24	6.22 $\pm$ 0.42	5.60 $\pm$ 0.89
Tumor	43.3 $\pm$ 4.88	46.2 $\pm$ 3.23	48.8 $\pm$ 0.31	74.0 $\pm$ 6.40



**Figure 2.** Tumor-to-normal tissue ratios of  $^{64}\text{Cu}$ -labelled DOTA-cBR96 (5:1), DOTA-cBR96 (10:1), DOTA-cBR96 (20:1) and DOTA-cT84.66 (20:1) in LS174T-bearing nude mice at 24-h post-injection. Error bars represent standard error of the mean.

tumour-targeting antibody. The stability of the  $^{64}\text{Cu}$ -monoclonal antibody DOTA chelate was 100% over a 48-h period in serum under physiologic conditions for both cBR96 and cT84.66. This suggests that the  $^{64}\text{Cu}$  is likely to remain bound to the monoclonal antibodies in circulation during the course of tumour targeting *in vivo*.

In LS174T-bearing nude mice, the biodistributions of these  $^{64}\text{Cu}$ -labelled monoclonal antibodies showed promising tumour-to-normal tissue ratios at 24 h post-injection. The tumour uptakes were uniformly high among all of the conjugates. There was no statistically significant difference between the  $^{64}\text{Cu}$ -DOTA-cBR96 conjugates, corroborating the uniformly high *in vitro* immunoreactivity results. This finding was also consistent with the results obtained by Meares and co-workers (Kukis *et al.*, 1995) evaluating  $^{67}\text{Cu}$ -labelled monoclonal antibody Lym-1. They determined that conjugates with up to 5 chelates per antibody showed no loss of immunoreactivity. At 24-h post-injection, tumour uptake of  $^{67}\text{Cu}$ -Lym-1 decreased, and liver and spleen uptake increased, with progressive modification of up to 11.4 chelates per antibody. However, this effect was insignificant at chelate/antibody ratios between 2.1 and 4.3. All conjugates evaluated in the present studies had DOTA substitution ratios in this range and displayed favourable tumour, liver and spleen uptakes.

Statistically significant increases in uptake were observed for  $^{64}\text{Cu}$ -DOTA-cT84.66, compared to  $^{64}\text{Cu}$ -DOTA-cBR96, in the liver, spleen and tumour. Antibody clearance occurs predominantly via the reticuloendothelial system, resulting in the deposition of some of the radioactive dose in the liver and spleen. The higher hepatic uptake of  $^{64}\text{Cu}$ -DOTA-cT84.66 observed in these studies may also be the result of expression of CEA receptors in the liver (Bajenova *et al.*, 2003), facilitating the uptake of circulating immune complexes. Tumour uptake of  $^{64}\text{Cu}$ -DOTA-cT84.66 was also significantly higher than that of  $^{64}\text{Cu}$ -DOTA-cBR96 at 24-h post-injection. The higher tumour uptake of  $^{64}\text{Cu}$ -DOTA-cT84.66 may be the result of the greater affinity of that monoclonal antibody for its antigen, higher expression of CEA in established LS174T xenografts or both. Dosimetry calculations for future RIT studies will take into account the difference in tumour uptake between the two antibodies, in order to deliver similar tumour-absorbed doses in the range of 5–15 Gy. The biodistribution of  $^{64}\text{Cu}$ -DOTA-cT84.66 reported here compares favourably with those previously reported for  $^{111}\text{In}$ - and  $^{90}\text{Y}$ -DOTA-cT84.66 in the LS174T mouse model (Williams *et al.*, 1998). With its high immunoreactivity and efficient  $^{64}\text{Cu}$  labelling characteristics, as well as its favourable biodistribution, the 10:1 DOTA-cBR96 conjugate appears to be optimal for direct therapeutic comparison with  $^{64}\text{Cu}$ -DOTA-cT84.66 in LS174T-bearing nude mice.

Evaluation of this two-antibody model in tumour-bearing mice may lead to further clinical investigation of both internalizing monoclonal antibodies and  $^{64}\text{Cu}$  in veterinary and human cancer patients. The Lewis<sup>x</sup> antigen has been demonstrated in biopsy samples of numerous canine carcinomas. These tumours represent aggressive, metastatic disease types with few effective options for systemic therapy. Copper-64 labelled radiopharmaceuticals offer the promise of specific targeting of lethal radiation to multiple sites in the body with minimal systemic side-effects. A single-chain construct of the variable binding domain of BR96, linked to a truncated, non-binding *Pseudomonas* exotoxin, has been evaluated clinically in canine carcinoma patients (Henry *et al.*, 1996). Clinical response or disease

stabilization was demonstrated in 7 of 12 dogs that received this immunotoxin. Intracellular delivery of  $^{64}\text{Cu}$  by internalizing antibodies like cBR96 might result in comparable or superior therapeutic efficacy. *Pseudomonas* exotoxin can only kill tumour cells that internalize the BR96 construct. In contrast,  $^{64}\text{Cu}$  offers the potential advantages of cross-fire killing due to  $\beta^-$  emission, as well as the possibility that internalization may effect cytotoxic mechanisms in addition to classical radiation damage. Elucidation of the unusual mechanisms of tumour cytotoxicity exerted by  $^{64}\text{Cu}$  at low radiation doses offers the promise of highly efficacious and minimally toxic RIT agents for veterinary and human cancer patients.

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