

# A preliminary study on treatment of human breast cancer xenografts with a cocktail of paclitaxel, doxorubicin, and <sup>131</sup>I-anti-epithelial cell adhesion molecule (9C4)

## ABSTRACT

Triple-negative breast cancer often has devastating outcomes and treatment options remain limited. Therefore, different treatment combinations are worthy of testing. The efficacy of a cocktail of paclitaxel, doxorubicin, and <sup>131</sup>I-anti-epithelial cell adhesion molecule (EpCAM) (9C4) to treat breast cancer was tested. Efficacy was tested with an MDA-MB-231 human breast cancer xenograft model. Anti-EpCAM (9C4) was demonstrated to bind to MDA-MB-231 human adenocarcinoma cells *in vitro*. Subsequently, mice-bearing MDA-MB-231 xenografts were treated with either <sup>131</sup>I-anti-EpCAM (9C4), unlabeled anti-EpCAM (9C4), paclitaxel, doxorubicin, or a cocktail of all of the agents. Tumor volume was measured for up to 70-day postinjection. Exponential regression was performed on tumor growth curves for each of the therapy groups. Statistical comparison of the growth constants  $\lambda$  of the regression models for each of the treatment groups with that of the cold antibody and control groups was done using extra sum-of-square F-tests. Biexponential clearance of <sup>131</sup>I-anti-EpCAM (9C4) was observed with biological clearance half-times of 1.14 and 17.6 days for the first and second components, respectively. The mean growth rate of the tumors in animals treated with a cocktail of all of the agents was slower than in those treated with unlabeled anti-EpCAM (9C4) ( $P = 0.022$ ). These preliminary data suggest that a cocktail of <sup>131</sup>I-anti-EpCAM (9C4), paclitaxel, and doxorubicin may be suitable for treating breast cancers with high expression of EpCAM.

**Keywords:** Chemotherapy, doxorubicin, epithelial cell adhesion molecule, iodine-131, monoclonal antibody, paclitaxel, radioimmunotherapy

## INTRODUCTION

Despite the relative success of standard chemotherapy agents, such as cyclophosphamide, doxorubicin, and paclitaxel, in significantly reducing the number and/or overall growth of cancer cells, such therapy often tends to leave a subpopulation of tumor cells unaffected. This is often because the cells that constitute the multidrug-resistant subpopulation are capable of actively pumping the drugs out of the cell. While chemotherapy provides an appropriate means of initially eradicating large numbers of cancer cells, there is a need for a second-line therapy option that is capable of treating cancer cells that survive chemotherapy. Treatment options that specifically target cancer cells over other cell types have been developed.

Radioimmunotherapy (RIT) is one such treatment strategy which achieved the United States Food and Drug

Administration approval for the treatment of hematologic malignancies including lymphoma.<sup>[1,2]</sup> RIT has shown to have more success against hematological malignancies as

**NAIIM S. ALI<sup>1,2</sup>, JOHN M. AKUDUGU<sup>1,3</sup>,  
ROGER W. HOWELL<sup>1</sup>**


<sup>1</sup>Division of Radiation Research, New Jersey Medical School, Rutgers, The State University of New Jersey, Newark, NJ 07103, <sup>2</sup>Department of Radiology, University of Vermont Medical Center, Burlington, VT 05401, USA <sup>3</sup>Department of Medical Imaging and Clinical Oncology, Division of Radiobiology, Faculty of Medicine and Health Sciences, University of Stellenbosch, Tygerberg 7505, South Africa

**Address for correspondence:** Prof. Roger W. Howell, Division of Radiation Research, New Jersey Medical School, Rutgers, The State University of New Jersey, Newark, NJ 07103, USA. E-mail: rhowell@rutgers.edu

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opposed to solid tumors. It has been hypothesized that the mechanism behind this discrepancy is not solely due to differences in radiosensitivity but also due to widely varying degrees of antibody (Ab) binding to the tumor cells.<sup>[3-6]</sup>

Identifying appropriate antigens to target remains a challenge in the development of immunotherapy agents. The epithelial cell adhesion molecule (EpCAM) is one possible target antigen that has been shown previously to be expressed significantly more on cancer cells than normal tissue.<sup>[7-11]</sup> Furthermore, RIT of human tumor xenografts with <sup>131</sup>I-anti-EpCAM (C215) has been encouraging,<sup>[12]</sup> and <sup>131</sup>I-anti-EpCAM (ING-1) has demonstrated good tumor targeting in patients.<sup>[13]</sup> Anti-EpCAM is also a key component of CELLSEARCH<sup>®</sup> technology which is used to identify circulating tumor cells in patients with metastatic breast, prostate, and colorectal cancer (Menarini Silicon Biosystems Inc., San Diego, CA, USA). Therefore, anti-EpCAM Abs remain as potentially viable vehicles for RIT.

The experimental studies undertaken in the present work sought to evaluate more about the ability of combined modality RIT to serve as a viable strategy in the treatment of solid tumors.<sup>[4,14,15]</sup> Cellular binding of anti-EpCAM (9C4) was quantified by flow cytometry in cultured human metastatic MDA-MB-231 cells. Mice-bearing human MDA-MB-231 tumors were treated with various combinations of <sup>131</sup>I-labeled anti-EpCAM (9C4), unlabeled anti-EpCAM (9C4), doxorubicin, and paclitaxel. The *in vivo* pharmacokinetics and therapeutic efficacy of combined modality RIT were evaluated.

## METHODS

### Radiolabeled antibody

The monoclonal Ab used in these studies was anti-EpCAM (9C4) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). This is a mouse monoclonal IgG<sub>2b</sub> raised against the human epithelial DU4475 breast carcinoma cell line that was derived from a metastasis on the skin. This particular Ab has been suggested for use in combination with other anti-EpCAM Abs for immunotherapy.<sup>[16]</sup>

To prepare the radiolabeled Ab, a quantity of 750 µg of anti-EpCAM (9C4) was labeled with 370 MBq of <sup>131</sup>I using the Iodogen technique. Iodogen tubes (Pierce Precoated Iodination Tubes, ThermoFisher Scientific, Rockford, IL, USA) were used for labeling. The Ab and Na<sup>131</sup>I (Perkin Elmer) were transferred to the Iodogen tube and incubated at room temperature for 75 min with periodic manual agitation of the tube every 15 min. As per the manufacturers' specifications, the reaction was terminated by removing the reaction

mixture from the precoated tube. Following removal of the reaction mixture, the reaction tube was rinsed with phosphate-buffered saline (PBS) and vortexed to recover residual product remaining in the tube. Labeled product was separated from free iodine with a PD-10 desalting column. Through previous trial labeling, it was determined that labeled product could be collected between the 6<sup>th</sup> and 12<sup>th</sup> fraction when eluted from the column in 0.5 mL fractions. The 7<sup>th</sup>, 8<sup>th</sup>, and 9<sup>th</sup> fractions were found to have a total activity of 109, 141, and 18 MBq, respectively, as measured with a dose calibrator. These fractions were pooled together to form the stock solution of <sup>131</sup>I-anti-EpCAM (9C4). To determine the fraction of activity bound to protein, 25 µL of these pooled fractions was added to 800 µL of cold acetone. Subsequently, 175 µL of albumin in PBS (1 mg/mL) was added to this mixture. The acetone solution was stored at -20°C for 1 h and was centrifuged at 15,000 g at 0°C for 10 min. Activity of both the pellet and supernatant was measured in the dose calibrator: 6.5 and 0.21 MBq, respectively, indicating that >97% was protein bound. The manufacturer specifications of the Iodogen labeling kit indicate a labeled protein recovery of >90%. The remaining stock solution was passed through a 0.22-µm pore size sterile filter before injection. The final concentration of the stock solution was 0.56 µg/µL, 0.138 MBq/µL of <sup>131</sup>I-anti-EpCAM (9C4).

### Cells

Human metastatic MDA-MB-231-luc-D3H2LN mammary carcinoma cells (Caliper Life Sciences, Waltham, MA, USA) were used for this study. This cell line does not express estrogen receptor and progesterone receptor, does not have HER-2/Neu amplification, and is therefore regarded as representative of triple-negative breast cancer.<sup>[17]</sup> These cells are generally sensitive to commonly used chemotherapy agents such as doxorubicin and paclitaxel, although the development of a drug-resistant cell line from this cell line has been described.<sup>[18]</sup> This cell line has previously shown a strong staining intensity for the anti-EpCAM (9C4) Ab,<sup>[16]</sup> the immunotherapy agent used for this study. These tumor cells have been transfected with a gene that expresses luciferase, which emits an abundance of visible photons upon exposing the cells to luciferin. Two different formulations of minimum essential medium (MEM) were used (MEMA for monolayer culture and MEMB for suspension culture), and they have been previously described in detail.<sup>[19]</sup> All media and supplements were obtained from Gibco, including fetal calf serum (catalog no. 10437, lot 539574). Cells were grown in MEMA as monolayers in Falcon 25-cm<sup>2</sup> tissue culture flasks (Becton Dickinson [BD], catalog no. 353082) at 37°C, and 5% CO<sub>2</sub> and 95% air and were subcultured twice weekly.

### Evaluation of distribution of cellular uptake of anti-epithelial cell adhesion molecule (9C4) *in vitro* with flow cytometry

MDA-MB-231-luc-D3H2LN cells were suspended in MEMB and treated with 0, 0.05, 0.1, and 0.2 µg/mL of PerCP Cy5.5-labeled anti-EpCAM (9C4) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated on a rocker roller for 2.5 h. The cells were washed twice with PBS, resuspended in 1 mL of PBS, passed five times through a 21-G needle, and immediately subjected to flow cytometric analysis. Cells were analyzed by flow cytometry using an LSR II flow cytometer (BD) equipped with a 488-nm laser. Emission spectra were captured within the wavelength transmitted by the 695/40 filter for PerCP Cy5.5-labeled anti-EpCAM (9C4).

### Tumor xenograft

All experimental procedures were in accordance with the institutional guidelines and approved by the Institutional Animal Care and Use Committee of the former University of Medicine and Dentistry of New Jersey, now Rutgers Biomedical Health Sciences. A total of  $2 \times 10^6$  MDA-MB-231-luc-D3H2LN cells were suspended in a solution of 25 µL MEMA with 25 µL Matrigel® Basement Membrane Matrix (BD, catalog no. 40234A) and injected orthotopically into the right fourth mammary fat pads of each of 25 4-week-old female NIH III *Lys<sup>t<sup>bg</sup></sup>*Foxn1<sup>nu</sup>Btk<sup>xid</sup> nu/nu nude mice (Charles River Laboratories, Wilmington, MA, USA). Tumors were allowed to grow for 8 weeks before treatment.

### Organization of animals into treatment groups

Eight weeks after inoculating the MDA-MB-231 cells (8 days before beginning treatment), 21 of the initially inoculated 25 animals were tumor bearing and were injected intravenously (*iv*) with 100 µL of 15 mg/mL D-luciferin (Xenogen, catalog no. XR-1001) in Dulbecco's PBS solution via the tail vein. Optical luminescence imaging of the mammary carcinoma xenograft was performed with a 1-s exposure and medium binning (IVIS® 200, Perkin Elmer, Waltham, MA, USA) beginning at 5 min postinjection of D-luciferin. Bioluminescence photon intensity emitted by the luciferase-expressing cells that comprise the tumor xenografts was quantified. Animals were ranked by bioluminescence photon intensity and placed into five groups to minimize differences in initial mean tumor size (proportional to intensity) between the groups: (1) <sup>131</sup>I-anti-EpCAM (9C4) alone (RIT), (2) <sup>131</sup>I-anti-EpCAM (9C4) with doxorubicin and paclitaxel (RIT + chemo), (3) unlabeled anti-EpCAM (9C4) alone (cold Ab), (4) doxorubicin and paclitaxel (chemo), and (5) nothing (control). Groups of 4, 4, 4, 5, and 4 mice were used for RIT, RIT + chemo, cold Ab, chemo, and control, respectively [Figure 1].

### Schedule of administrations

Previous studies demonstrated improved therapeutic effect when chemotherapy was administered after RIT.<sup>[20,21]</sup>

Accordingly, in the present study, chemotherapy agents were given 1-day post-RIT. Thus, the groups were administered the various agents according to the schedule that follows.

### Administration of radioimmunotherapy agent

On day 0, the mice in the two groups receiving RIT (RIT and RIT + chemo) each received 12.95 MBq of <sup>131</sup>I-anti-EpCAM (9C4) in 100 µL PBS delivered *iv*. The 12.95 MBq falls in the range of administered activities used in published studies with similar animal models.<sup>[3,20-23]</sup> These animals were subsequently imaged using a portable gamma camera equipped with a high-energy collimator; a 20% energy window around 364 keV was used [Figure 2]. Three animals in the RIT group were determined to have received “missed injections” based on gamma camera imaging and were thus boosted with a second injection of 11.88 MBq <sup>131</sup>I-anti-EpCAM (9C4) in 100 µL PBS on day 1 [Figure 1]. The boost injection was administered intraperitoneally (*ip*). On day 0, the cold Ab group received 50 µg of unlabeled anti-EpCAM (9C4) in 100 µL PBS delivered *iv*. The remaining two groups (chemo, control) each received 100 µL PBS injected *iv*.

### Administration of chemotherapy agents

On day 1, the two groups receiving chemotherapy (chemo and RIT + chemo) each received a cocktail of 300 µg paclitaxel and 50 µg doxorubicin in a solution of 80 µL PBS, 10 µL cremophor EL, and 10 µL ethanol, delivered *ip*. Doses and preparation of chemotherapy agents used were consistent with those outlined in the previous studies.<sup>[20-22,24]</sup> The remaining three groups (RIT, cold Ab, and control) all received a solution of 80 µL PBS, 10 µL cremophor EL, and 10 µL ethanol, delivered *ip*.

### Evaluation of whole-body pharmacokinetics of <sup>131</sup>I-anti-epithelial cell adhesion molecule (9C4)

Whole-body counts for the animals receiving RIT and RIT + chemo were measured with the portable gamma camera immediately following treatment, as well as 1, 3, 7, and 21 days posttreatment. Animals were secured to the camera using adhesive tape. Each animal was counted for 10 min and background counts for the same period were subtracted.

### Evaluation of therapeutic effect through measurement of tumor volume

To evaluate the therapeutic effect of the various treatments, tumor volume was measured 8 days before treatment, as well as 6, 14, 20, 27, 34, 41, 49, and 74 days posttreatment. The tumor volume *V* was estimated from orthogonal caliper measurements of the equatorial diameters (*a*, *b*) of the ellipsoid-shaped tumor according to  $V \sim 0.4 a b^2$ , where  $b \leq a$ .<sup>[25]</sup>

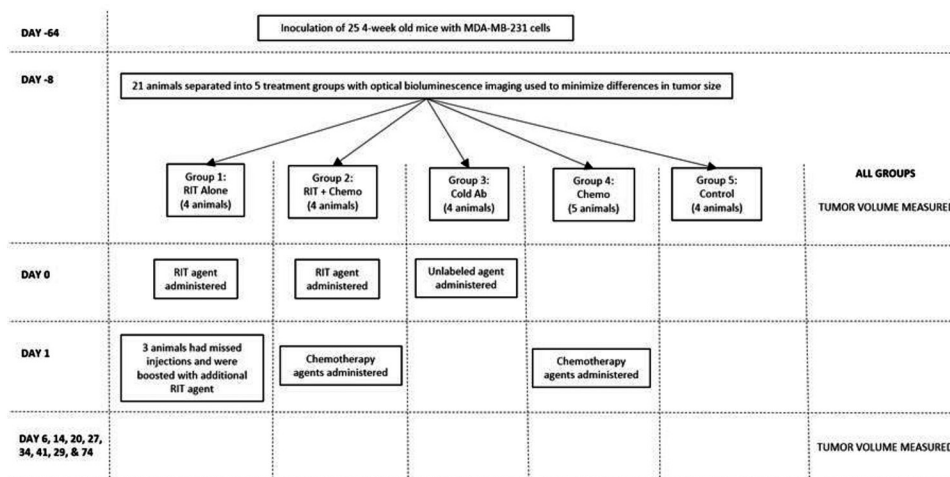


Figure 1: Timeline of study events outlining inoculation of animals with MDA-MB-231 cells, treatments, and tumor measurements

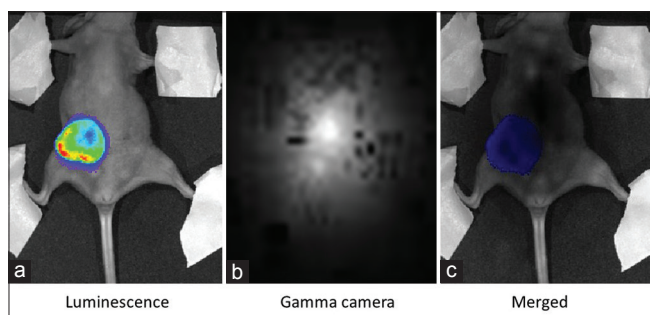


Figure 2: Imaging performed in a representative animal: (a) optical luminescence imaging of the luciferase-expressing cells comprising the mammary carcinoma xenograft was performed postinjection of D-luciferin. (b) Gamma camera imaging was performed after tail vein injection of 12.95 MBq <sup>131</sup>I-anti-EpCAM (9C4). (c) Fused image of optical luminescence image and gamma camera image. EpCAM: Epithelial cell adhesion molecule

## RESULTS

### Distribution of cellular uptake of anti-epithelial cell adhesion molecule (9C4)

Flow cytometry side scatter versus forward scatter data were gated to exclude debris [Figure 3]. Figure 4 shows flow cytometry histograms of the fluorescence intensity of cells that were treated with 0, 0.05, 0.1, and 0.2 μg/mL of PerCP Cy5.5-labeled anti-EpCAM (9C4). Increasing concentration of anti-EpCAM (9C4) yielded increasing levels of the intensity on flow cytometry analysis.

### Whole-body clearance of <sup>131</sup>I-anti-epithelial cell adhesion molecule (9C4)

Whole-body activity measurements were averaged for each measurement date with the three “missed injections” removed from the average. The averaged data were least-squares fitted to a two-component exponential function using SigmaPlot 12 [Figure 5],

$$A = ae^{-\frac{0.693t}{T_{e,1}}} + be^{-\frac{0.693t}{T_{e,2}}}$$

where  $A$  is the whole-body activity in counts per minute (CPM),  $a$  and  $b$  are fitted parameters, and  $T_{e,1}$  and  $T_{e,2}$  are the effective clearance times for the first and second components. The fitted values were  $2.83 \times 10^6$  CPM,  $5.01 \times 10^5$  CPM, 1.0 day, and 5.5 days, respectively. The effective clearance half-time  $T_e$  is related to the physical half-life  $T_p$  and biological clearance half-time  $T_b$  according to the relationship  $1/T_e = 1/T_b + 1/T_p$ . Therefore, with the physical half-life of <sup>131</sup>I being 8.03 days, solving the equation above for  $T_b$  gives biological clearance half-times of 1.14 days and 17.6 days for the first and second components, respectively.

### Evaluation of therapeutic effect

Therapeutic effect of the various treatments was assessed through measurement of tumor volume using calipers as previously described. Tumor volumes were averaged for each treatment group on each measurement date and plotted [Figure 6]. Data demonstrate a large variation of tumor volumes. Nonlinear regression with an exponential growth equation was performed for each group using PRISM 6 software (GraphPad PRISM 6; La Jolla, CA, USA):  $V(t) = V_0 \exp(\lambda t)$ . PRISM 6 was used to compare the growth constants  $\lambda$  of the regression models for each of the treatment groups with that of the cold Ab and control groups, using extra sum-of-square F-tests [Table 1]. The difference between the RIT + chemo and cold Ab groups was found to be statistically significant ( $P = 0.022$ ).

## DISCUSSION

### Study limitations

Between four and five animals were used for each of the treatment groups. Heterogeneity within the treatment groups in terms of both the initial and treated tumor volumes led

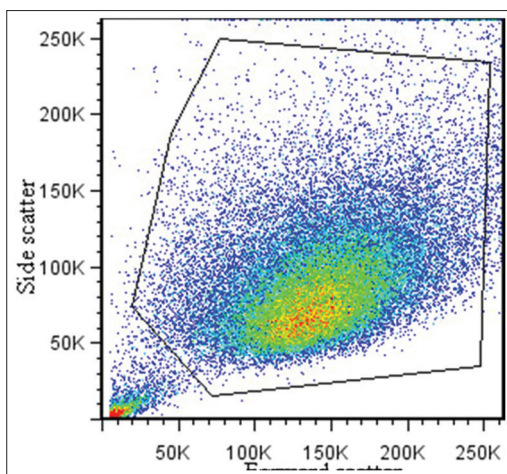


Figure 3: Flow cytometry analysis of side scatter versus forward scatter for MDA-MB-231-luc-D3H2LN cells treated with PerCP Cy5.5-labeled anti-EpCAM (9C4). EpCAM: Epithelial cell adhesion molecule

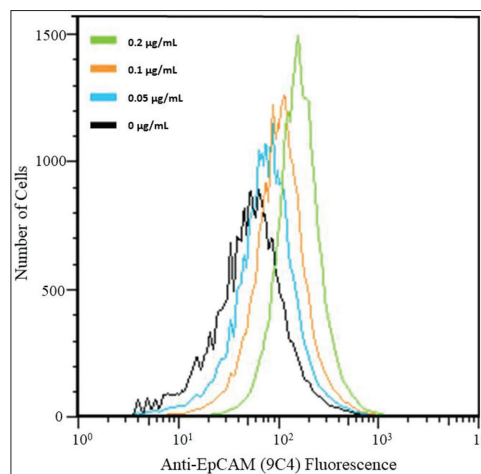


Figure 4: Distribution of cellular binding of anti-EpCAM (9C4) by MDA-MB-231-luc-D3H2LN cells in suspension culture: flow cytometry histograms of cellular fluorescence intensity after treatment with 0, 0.05, 0.1, and 0.2 µg/mL PerCP Cy5.5-labeled anti-EpCAM (9C4). EpCAM: Epithelial cell adhesion molecule

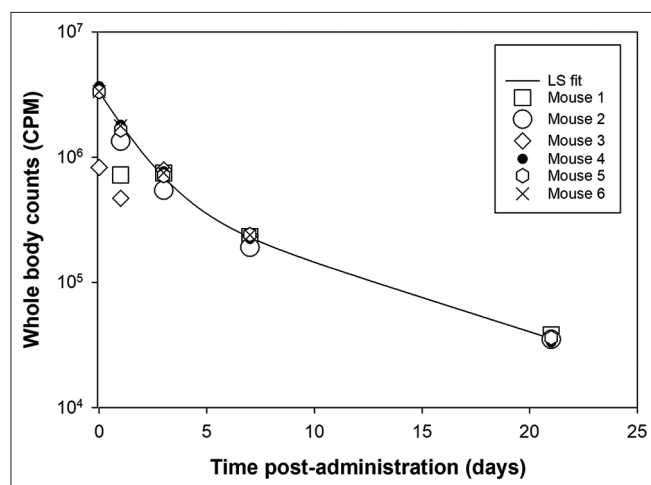


Figure 5: Whole-body clearance of <sup>131</sup>I-anti-EpCAM (9C4). The whole-body counts per minute were recorded as a function of time postinjection of 12.95 MBq <sup>131</sup>I-anti-EpCAM (9C4). Each symbol type represents a different animal. Note that the low values on day 0 represent three “missed injections;” these animals received a second injection of 11.88 MBq after the whole-body count measurement on day 1; note that this resulted in the whole-body activity being nearly the same in all animals on day 3. EpCAM: Epithelial cell adhesion molecule

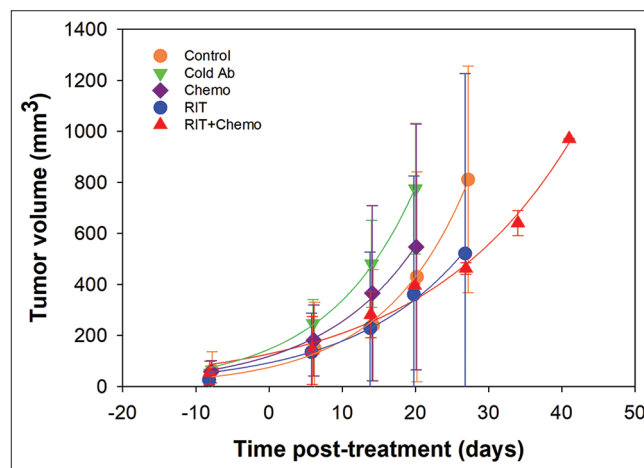


Figure 6: Average tumor volume as a function of time posttreatment. Animals that died were not included in the averages following their date of death. For treatment groups in which animals were euthanized due to the presence of a tumor volume of 1000 mm<sup>3</sup>, average volumes were not calculated past the date of euthanizing the first animal to be euthanized in the group. Error bars represent standard error of the mean. Curves are exponential regression models

to large confidence intervals in mean tumor volumes for each of the treatment groups. Future studies utilizing larger treatment groups may be able to mitigate the effects of such expected heterogeneity to draw more decisive conclusions.

For three of the animals, tail injections of the RIT agent were determined to be missed based on gamma camera imaging performed after treatment. While these animals were supplemented with additional RIT agent, evaluation of potentially missed injections could not be performed in the treatment group that received the unlabeled immunotherapy agent (cold Ab). Therefore, the potential therapeutic effect of the unlabeled immunotherapy agent is difficult to assess

based on this study. A future study may benefit from labeling of immunotherapy agents with nontherapeutic radiotracers such as technetium-99m to be able to determine appropriate agent administration.

Several animals in each of the different treatment groups were found dead at various points after cancer cell inoculation including before treatment and after treatment. The causes of these unexpected deaths remain unclear although extensive tumor burden may be one possible etiology. Animals that died were not included in the averages following their date of death. Future studies may benefit

**Table 1: Tumor growth. Exponential regression of tumor volume versus time ( $V(t) = V_0 \exp[\lambda t]$ ) for the five treatment groups with comparison to control and cold antibody in the form of unlabeled anti-epithelial cell adhesion molecule (9C4) groups\***

Treatment group	$\lambda$ (/days)	SE of $\lambda$	$V_0$ (mm <sup>3</sup> )	P versus control	P versus cold Ab
Control	0.087	0.029	76		
RIT + chemo	0.050	0.004	123	0.12	0.022
RIT	0.068	0.040	86	0.70	0.69
Cold Ab	0.084	0.018	146		
Chemo	0.077	0.038	118	0.84	0.87

\*RIT: Radioimmunotherapy with <sup>131</sup>I-anti-EpCAM (9C4); Ab: Antibody in the form of unlabeled anti-EpCAM (9C4); EpCAM: Epithelial cell adhesion molecule; SE: Standard error; chemo: Chemotherapy

from starting treatment at an earlier time course after tumor cell inoculation.

### Significance and implications of results

This study evaluated the ability of <sup>131</sup>I-anti-EpCAM (9C4), in combination with chemotherapy, to treat human mammary adenocarcinoma in a mouse model. The measured whole-body clearance data for <sup>131</sup>I-anti-EpCAM (9C4) suggest good radiochemical integrity *in vivo* as evidenced by the 1.14 and 17.6 days biological half-times for the first and second components of the whole-body clearance, respectively [Figure 5]. These are consistent with published pharmacokinetic data for murine IgG<sub>2b</sub> Abs.<sup>[26]</sup> When combined with chemotherapy, this translated to tumor control as evidenced by a significantly ( $P = 0.022$ ) lower exponential tumor growth constant for animals treated with RIT + chemo compared to those treated with cold Ab [Table 1]. However, large variations in tumor sizes both at the start of and after treatment as well as issues with tail vein injection of the immunotherapy agent and unexpected animal deaths temper the significance of this finding. Yet, the significant tumor response is supported by the *in vitro* flow cytometry data which indicate cellular binding of anti-EpCAM (9C4) to the MDA-MB-231 tumor cells [Figure 4]. Nevertheless, all animals eventually succumbed to the tumor, a common problem in cancer therapy. This may be due, in part, to the lognormal nature of the cellular uptake profile observed in Figure 4. Such nonuniform distributions of uptake of radiopharmaceuticals and chemotherapy agents can have a detrimental impact on biological response of the tumor.<sup>[4,6,27-29]</sup>

### CONCLUSION

Combined modality RIT remains a promising approach in the treatment of mammary adenocarcinomas. While *in vivo* imaging and measurement of therapeutic effect in this study indicate mixed results, *in vitro* cellular binding and *ex vivo* activity measurements support selective targeting

by <sup>131</sup>I-anti-EpCAM (9C4). Notably, EpCAM is a good marker for circulating tumor cells. Further studies evaluating radiolabeled agents such as <sup>131</sup>I-anti-EpCAM (9C4) are needed to advance the role of RIT agents in the treatment of mammary adenocarcinoma.

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### Conflicts of interest

John M. Akudugu and Roger W. Howell hold United States patents on related technology (US Patent No. 8,874,380, US Patent No. 9,623,262, and US Patent No. 9,804,167 B2).

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