Award Number: DAMD17-02-1-0613

TITLE: Optical Imaging of Mammaglobin Expression of Breast Cancer

PRINCIPAL INVESTIGATOR: Samuel Achilefu, Ph.D.

CONTRACTING ORGANIZATION: Washington University
St. Louis, Missouri 63110

REPORT DATE: May 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2003	Annual (1 May 2002 - 30 Apr 2003)

4. TITLE AND SUBTITLE
Optical Imaging of Mammaglobin Expression of Breast Cancer

5. FUNDING NUMBERS
DAMD17-02-1-0613

6. AUTHOR(S)
Samuel Achilefu, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Washington University
St. Louis, Missouri 63110

E-Mail: achilefus@mir.wustl.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Mammaglobin (MMG) is a breast-specific glycoprotein that is over-expressed in 80% of all primary and metastatic breast cancer. Despite its uniqueness as a breast-specific cancer marker, the utility of MMG in breast cancer imaging has not been explored. Consequently, the goal of this project is to evaluate the potential of imaging the expression of MMG in established human breast cancer models. TO accomplish this goal, we labeled polyclonal and monoclonal anti-MMG antibodies with a near infrared fluorescent probe for optical imaging and ^{64}Cu-DOTA for positron emission tomography (mPET). Preliminary results indicate that the mPET imaging with ^{64}Cu-DOTA-anti MMG monoclonal antibodies showed predominant liver uptake in mice. In contrast, the fluorescent-labeled derivative of polyclonal MMG antibody primarily accumulated in the tumor, kidneys and liver. We have also developed a tumor model with increased expression of MMG. Our preliminary results appear to support the hypothesis that targeting the expression of MMG will enhance the localization of breast cancer tumors.

14. SUBJECT TERMS
Optical imaging; optical contrast agents; radiopharmaceuticals; Positron emission tomography; mammaglobin; tumor targeting

15. NUMBER OF PAGES
15

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

The observation that the expression of mammaglobin (MMG) is uniquely associated with human breast and, more importantly, up-regulated in breast cancer provides an opportunity to develop a novel approach to image breast cancer by targeting MMG expression in vivo. This concept is based on the premise that the concentration of secreted MMG will be up-regulated in the tumor-microenvironment, thereby facilitating the localization of the breast cancer and possible metastasis. This will require the use of highly sensitive methods that can detect low levels of diagnostic protein expressions at the molecular level. This criterion can be satisfied by the use of optical or nuclear imaging method. Therefore, this project focuses on the preparation of anti-MMG antibodies, native glycosylated MMG protein, non-glycosylated synthetic MMG, labeling the biomolecules with optical or nuclear probes, and evaluating the possibility of localizing tumors by either or both of these methods. Our preliminary findings indicate that this approach is feasible.

BODY OF REPORT

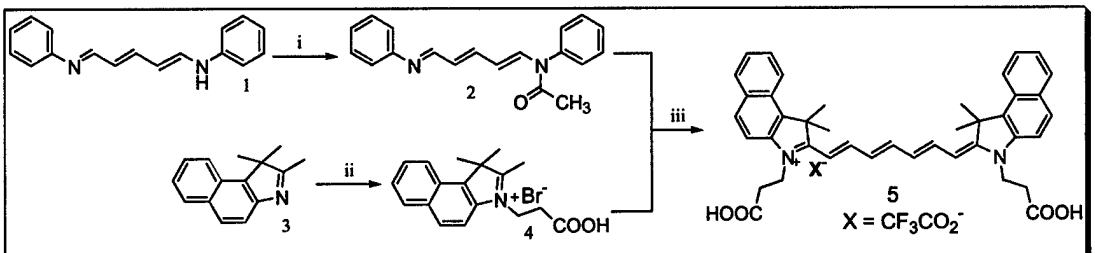
As described in our approved Statement of Work, the scope of this project requires that several components of the work run concurrently. Thus, in addition to accomplishing the stated goals of the first 12 months of this project, we have also started to perform some of the tasks (biodistribution and imaging studies) slatted for the latter part of the study. Integrating most components of the work enable us to focus on the aspects of the project with potential high impart for breast cancer management. The following activities were approved for this report period:

TASK 1(a)

Synthesize and characterize two mouse anti-mammaglobin monoclonal antibodies (AMABs) conjugates of cypate, a near infrared indotricarbocyanine optical probe (Months 1-9).

(i) Synthesis of near-infrared fluorescent probe, cypate. Cypate is not commercially available and was prepared in our laboratory by modifying the method we described previously (1). We have improved the synthesis method, which is summarized below. A mixture of 1,1,2-trimethyl-[1H]-benz[e]indole (10.0g, 47.8mmole) and 3-bromopropanoic acid (7.3g, 47.8mmoles) in 1,2-dichlorobenzene (50 mL) was heated with stirring at 110 °C for 18 h. After the resulting mixture was cooled to room temperature, the precipitated was collected by filtration, triturated with DCM thoroughly, and dried under vacuum to give the 15.2 g (88%) of the intermediate product, **1,1,2-trimethyl[1H]-benz[e]indole-3-propanoic acid** [ESI-MS: observed for $[MH]^+$ 281.31].

A solution of Ac_2O (1.20g, 11.75 mmol) in dichloromethane (DCM, 5 mL) was added dropwise to a cooled, stirring suspension of glutaconaldehyde dianilide monohydrochloride (2.84g, 9.97 mmol) and diisopropylethylamine (DIEA, 2.60 g, 20.11 mmole) in DCM (20 mL). The resulting clear solution was stirred for another 3 h and concentrated under vacuum. The residue was dissolved in methanol (5.0 mL) was added dropwise to a refluxing solution of **1,1,2-trimethyl[1H]-benz[e]indole-3-propanoic acid** (**4**; 10.0g, 27.62 mmol) prepared above and sodium acetate (3.9g, 47.54 mmol) in methanol (50 mL). The mixture was refluxed for 16 h and concentrated. The residue was washed with ethyl acetate, 5% HCl solution, and ethyl acetate. The crude product was further purified by recrystallization from acetonitrile/water (3:7) to afford 4.3g (61%) of cypate [**5**; analytical HPLC retention time: 20.62 min; ESI-MS: observed for $[MH]^+$ 625.34]. Scheme 1 below summarizes the reaction procedure.



Reagents and Conditions: i. Ac_2O/DIEA/DCM/1h; ii.$BrCH_2CH_2COOH$/Dichlorobenzene/115°C/16h; Iii. CH_3OH/reflux/16h.

Scheme 1. Synthesis of Cypate

Figure 1 below shows the absorption and emission spectral properties of cypate in 25% aqueous dimethylsulfoxide (DMSO). Note that the corrected fluorescence spectrum in blood typically has an emission λ_{max} at about 840 nm.

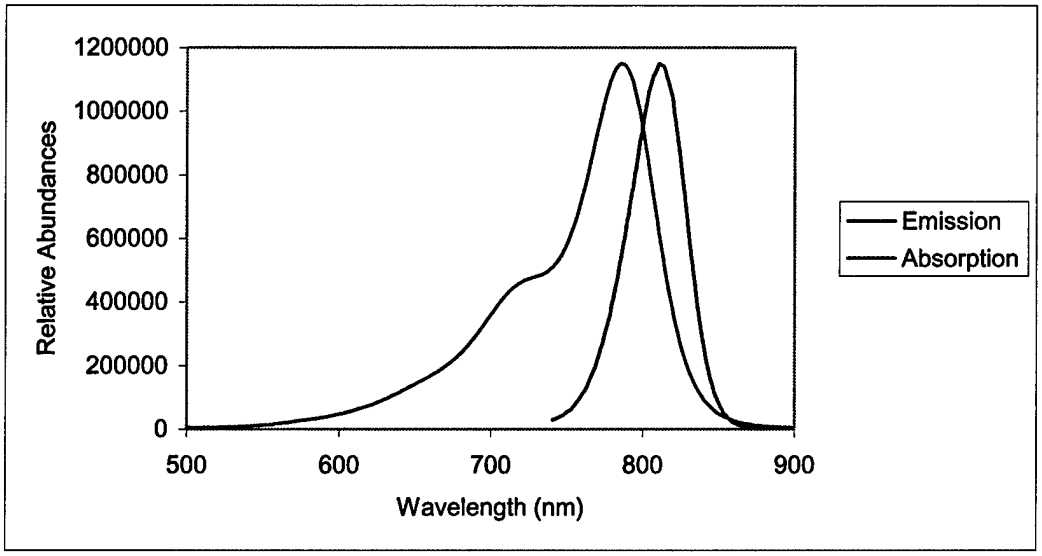


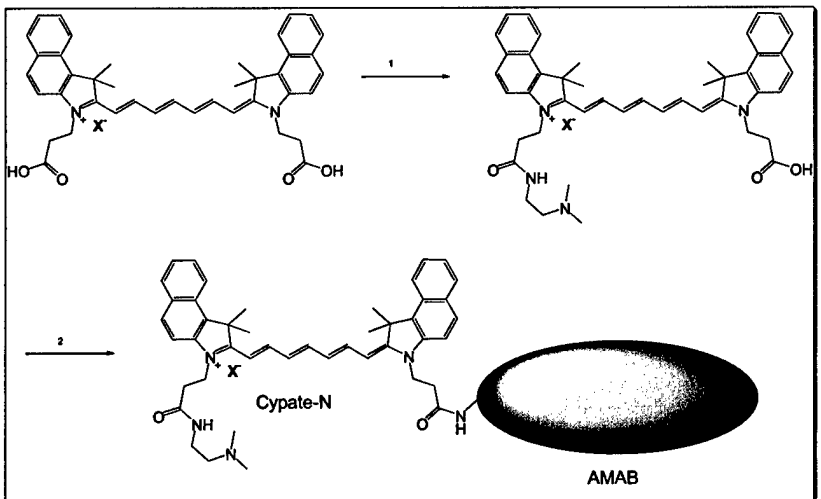
Figure 1: Absorption and Emission properties of cypate

(ii) **Synthesis of the novel Cypate derivative (Cypate-N)**: Cypate has two carboxyl groups that can facilitate protein cross-linking and polymerization. To circumvent this problem, we have synthesized a new cypate derivative that will be published in the near future. The new compound only has one activatable carboxyl group the method for its synthesis from cypate is summarized in Scheme 2. The crude product was purified by HPLC and identified by ESI-MS.

(iii) **Antibody purification:** The antibody was purified from serum using the Bio-Rad Serum IgG purification kit per the kit instructions. Briefly, the DEAE Affi-Gel blue column was prewashed with two column volumes of 0.01 M acetic acid, pH 3.0 containing 1.4 M NaCl and 40% (v/v) isopropanol and two column volumes of application buffer (0.02 M Tris-HCl, pH 8.0 containing 0.028 M NaCl). After equilibrating the column with application buffer, the sample was applied to the

column and eluted with application buffer. 5 ml fractions were collected. Fractions containing protein, as determined by measuring absorbance at 280 nm, were pooled. Purified protein was recovered by lyophilization.

(iv) **Conjugation of cypate with anti-MMG monoclonal antibody:** The anti-MMG monoclonal antibody (AMAB) used in this study was supplied by Corixa Inc, as promised in the proposal Support Letter from the company. The AMAB binds to MMG via different linear and conformational epitopes. The antibody has been shown to be specific for detecting MMG via Western blot analysis, ELISA, and FACS. Conjugation of cypate to the AMAB was accomplished by reacting a mixture of pre-activated cypate-N with the AMAB in DMSO-water for 16 h (see Scheme 2). The crude product was concentrated by lyophilization and purified by gel filtration. Different fractions were collected and identified by UV-Vis.



Reagents and conditions: 1. EDCI/HOBT/DMF/DCM, 8h; 2. EDCI/DMSO/Protein, 16h.

Scheme 2. Labeling AMAB with a new a mono-carboxylate containing Cypate

TASK 1(b)

Label mouse AMAB with ^{111}In and ^{64}Cu chelates; purify and characterize radiolabeled AMAB (Months 3-12).

(i) Bioconjugation of DOTA with AMAB: We modified existing methods for labeling proteins for this reaction. The concentration of AMAB protein was determined by UC-vis at 280 nm. A mixture of DOTA, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl salt, and N-Hydroxysulfosuccinimide sodium salt in water was stirred for 2 h at 4 °C, followed by adjusting the pH to 7.5 units by adding 0.2 M Na_2HPO_4 buffer. AMAB was added to the activated DOTA and the mixture was rota-mixed at 4 °C overnight. The solutions were transferred to new Centricon filter tube and loaded onto centrifuge for 1 hour to get rid of unreacted small molecules such as DOTA, EDC, SNHS, etc. This process was repeated several times to make sure that the small molecular weight reagents are completely removed. The concentration of the DOTA-AMAB was determined by fast protein liquid chromatography at 280 nm (conjugation yield is 40%).

(ii) **^{64}Cu Radiolabeling of DOTA-AMAB:** Radiolabeling of the DOTA conjugate was achieved by adding a solution of 10.6 mCi of $^{64}Cu(OAc)_2$ (200 μL in 0.1M NH_4OAc, pH 6.5. 11:05 am) to 400 μL of DOTA-MMG solution (159 μg). The resulting solution was incubated at 43°C in a water-bath for 2 h. DTPA (10 mM) was added to the solution to challenge the non-specifically bound ^{64}Cu (10 min at RT). The conjugate was purified by Bio-Spin 6 column and the fast protein liquid chromatography results indicated that the second separation and DTPA challenge was not necessary. Over 95% radiochemical purity was achieved after the first separation, with a radiolabeling yield of 84%.

TASK 1(c)

Synthesize native MMG glycoprotein in bacteria and non-glycosylated synthetic MMG proteins by segment condensation peptide synthesis; labeled with optical and radioactive probes (Months 2-20).

(i) **Preparation of bacterially-expressed MMG:** MMG was expressed in bacteria using the BAD/His expression vector. The protein was purified on a nickel-

chelating resin and the mammaglobin was separated from the fusion protein using enterokinase. Our carboxy terminal peptide antibody recognizes the bacterially expressed MMG protein. We are able to produce milligram quantities for use in this project.

(i) **Preparation of non-glycosylated synthetic MMG:** To accomplish the segment synthesis of non-glycosylated MMG, we divided the 93 amino acid MMG sequence into 4, based on the published homology of the protein with other gene family members (2). The structures of the protein segments are:

MKLLMVLMLAALSQHCYA

GSGCPLLENVISKTINPQVSKTEYKELLQEF

IDDNATTNAIDELKECF

LNQTDETLSNVEVFMQLIYDSSLCDLF

Synthesis of these peptides is in progress. Each peptide segment will be labeled and tested for possible binding to AMAB. Identification of a peptide motif that has similar binding affinity to AMAB as MMG will facilitate the evaluation of the role of MMG in breast cancer pathogenesis. Such compounds will also be used to assess the assess the possible existence of MMG receptors in vivo.

TASK 2(a)

Characterize and classify six cell lines as MMG-positive and MMG-negative cancer cells. These include four human breast cancer cell lines, MDA-MB-415, MDA-MB-361, MDA-MB-451, and MCF-7, and two established rat acinar pancreatic cell lines, CA20948 and AR42-J (Months 6-10).

We have positively identified the expression of MMG in MDA-MB-415, MDA-MB-361, and MCF-7. Protein detection and expression of mammaglobin in breast cancer cell lines is well documented in the literature (2-4), and illustrated in this report with the MDA-MB-415 tumor model. The MMG protein has two consensus

N-glycosylation sites and tunicamycin-treated MDA-MB-414 breast cancer cells abolished the detection of the mature form of MMG (data not shown). The predicted size of the MMG protein from its cDNA is ~6 kD. Figure 2 shows the detection of MMG protein in the cell culture supernatant of MDA-MB-415. To confirm that the observed size of the mature mammaglobin protein is due to N-linked glycosylation, we treated culture supernatants from the MDA-MB-415 breast tumor cell line with N-glycosidase F. As shown in Figure 3, incubation of the 21 kD protein recognized by the mammaglobin specific antibody (lane 1), resulted in the appearance of the 14 kD form observed in cell lysates (lane 2). Upon further incubation, the predicted 6 kD band was also observed (Figure 3, Lane 3).

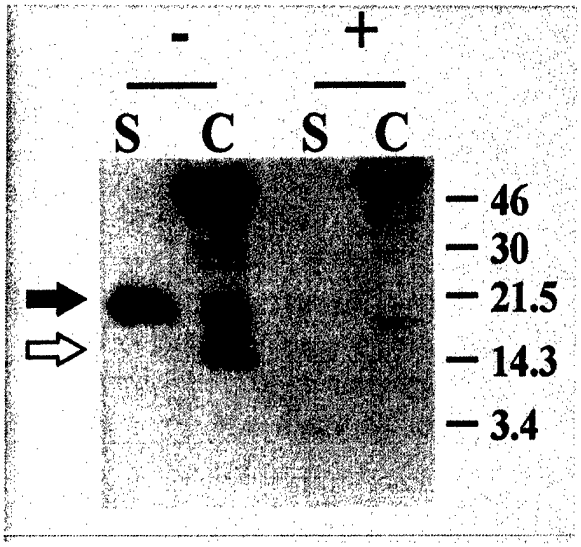


Figure 2. Detection of mammaglobin protein in the culture supernatant of the human breast cell line MDA-MB-415. Under reducing conditions, a ~21 kD band is detected by Western blot analysis in the culture supernatant (S) and a ~14 kD precursor form is observed in the cell lysate (C). The right panel (+) is identical to the left panel (-) except that competing peptide was added to demonstrate specificity of the peptide antibody generated to the mammaglobin protein

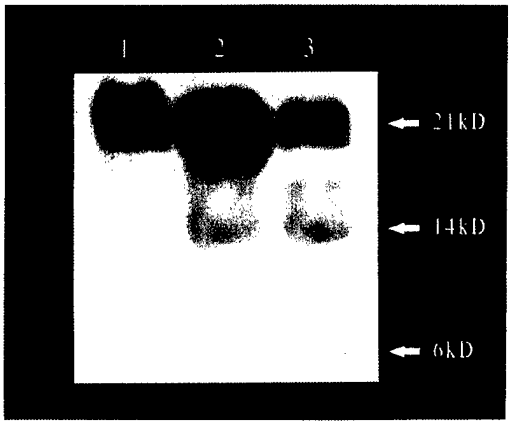


Figure 3: Treatment of native mammaglobin with N-Glycosidase F. Culture supernatants from the breast cancer cell line, MDA-MB-415were treated with: no enzyme (lane 1); 15 minutes with N-Glycosidase F (lane 2); or 30 minutes treatment (lane 3). This Western blot was run under reducing conditions.

The CA20948 cell line was evaluated for mammaglobin expression using RT/PCR analysis. This cell line was negative for a mammaglobin specific band (Fleming, personal communication) and can serve as a negative control for our studies.

TASK 3

The in vivo and imaging studies were scheduled to start 18 months from the Award date but we started this earlier to facilitate the integration of imaging information with in vitro studies. Below are the results of our preliminary imaging studies.

(i) **Optical Imaging of cypate-AMAB in mice:** A simple non-invasive *in vivo* continuous wave fluorescence imaging apparatus employed to assess the localization and distribution of contrast agents has been previously described (5, 6). Briefly, two de-focusing lenses were each placed in front of two laser diodes to expanded the beam such that the whole mouse was illuminated. The diodes emit radiation at 780 nm, which excites cypate-AMAB conjugate. The lasers generated a nominal 50 mW of incident power but the power at the output of the bundle was approximately one-half of the input power.

We used a Princeton Instruments CCD camera to capture the emitted light and an interference filter was placed in front of the CCD (830 nm) to reject unwanted photons. Images were acquired and processed using WinView software from Princeton Instruments. Typically, an image of the animal was taken pre-administration of contrast agent. Subsequent images were taken post administration of the agent, all performed with the mouse in a stationary position. Data analysis consisted of subtracting (pixel by pixel) the pre-administration image from the post administration images, and displaying the results in false color. For images taken several hours post administration, the animals were removed from the sample area, returned to their habitat, and then brought back to the sample area. Background subtraction was not performed for these measurements.

Figure 4 shows that the AMAB fluorescent probe was retained in the tumor and the kidneys, relative to other organs. The ex-vivo image (Figure 5) of selected organ parts confirms the whole-body optical image. The results is somewhat surprising because such large bioconjugates are typically excreted by the liver and not the kidneys and suggest a potential active transport system that may be associated with MMG receptor. We plan to validate this preliminary data by performing additional optical imaging experiments and histology.

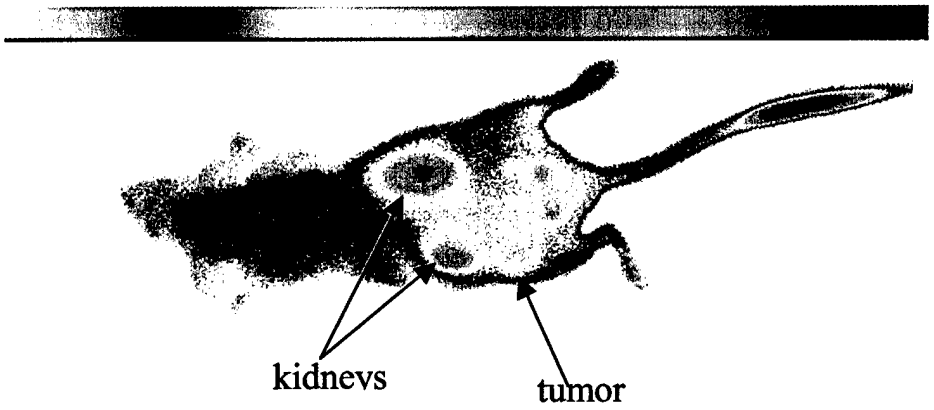


Figure 4: Cypate-N AMAB conjugate injected in MDA-MB-361 tumor in nude mouse 20 hour post administration

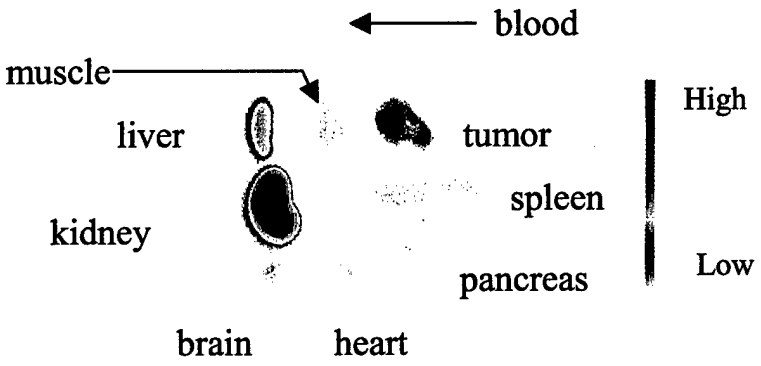
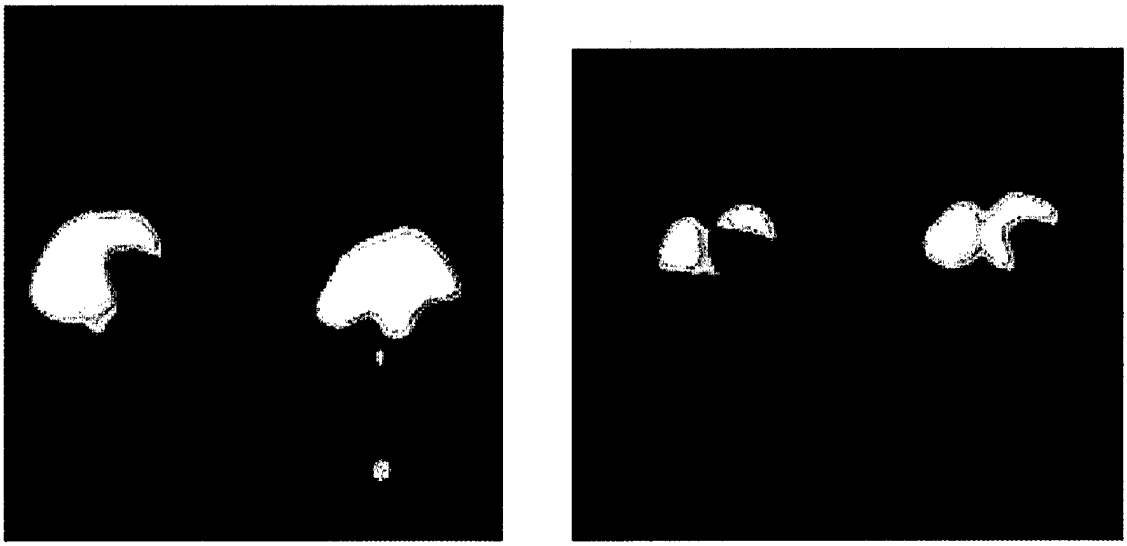


Figure 5: Ex vivo tissues at 20 hours post administration Cypate derivative/polyclonal antibody in mda-mb-361 tumor in nude mouse

(ii) **MicroPET imaging of ^{64}Cu-DOTA-AMAB**: Cells from a newly stably transfected human mammary tumor cell line (MC7-MAM) were injected into the left flank of four nude mice and after 7 days, the rodents were used for microPET imaging to evaluate the distribution of the DOTA-AMAB in mice. About 1.1 mCi/125-μL (~30 μg DOTA-MMG) was injected into each rodent via the lateral vein. Representative images are shown in Figure 6. The tumor was not palpable at the time of imaging and the radiolabeled AMAB was primarily retained in the kidneys. We could not confirm if the hot spots observed at the right abdominal region in Figure 6 correlates with metastatic tumor. More studies are planned in the near future.



(a) (b)

Figure 6: MicroPET image (Coronal view) of mice injected with ^{111}In-DOTA-MMG mAb at (a) 3 h and (b) 24 h postinjection. Radiolabeled probe accumulates in liver.

KEY RESEARCH ACCOMPLISHMENTS

(1) We have prepared the first near infrared and radiometal chelate of anti-MMG antibodies.

(2) We demonstrated, for the first time that labeling anti-MMG polyclonal antibody with a near infrared fluorescent probe enables the detection of MMG expressing tumors by optical imaging. The surprising renal excretion of

such large near infrared probe-antibody bioconjugate may reflect a receptor-mediated active transport into the kidneys

(3) MicroPET imaging with ^{64}Cu-DOTA conjugate of mAb in nude mice show that the antibody is excreted by the liver. Experiments are planned to evaluate the biodistribution in MMG-positive tumor-bearing mice.

REPORTABLE OUTCOMES

Although our preliminary data are exciting, further studies are needed to validate the results. We believe that within the next 10 months, peer-reviewed papers will be published. In addition, we may have to patent MMG-mediated imaging of breast cancer because of its potential to revolutionize breast cancer imaging by optical methods. Unfortunately, our results have to be validated before an informed decision could be made. Because of this uncertainty, we have authorized the release of this report to the public. The concept of imaging MMG expression in breast cancer were presented at the following invited talks by the PI:

1. S. Achilefu: Design of optical imaging agents **[Invited Speaker]**. NIH Workshop on Imaging the Pancreatic Beta Cells, Bethesda, MD (April 21-22, 2003).

2. S. Achilefu: Imaging and monitoring therapeutic response of tumors by optical methods **[Invited Speaker]**. Washington University Small Animal Imaging Symposium, St. Louis, MO (April 14, 2003).

3. S. Achilefu: Optical imaging – instrumentation and methods **[Invited Speaker]**. Siteman Cancer Center Oncologic Seminar Series, Washington University Medical School, St. Louis, MO (December 9, 2002)

4. S. Achilefu: Optical contrast agents for tumor imaging **[Invited Speaker and panelist]**. NIH Workshop on Optical Imaging, Bethesda, MD (September 26-27, 2002).

5. S. Achilefu: Somatostatin beacon: a reliable model for targeted delivery **[Invited Speaker]**. The Society for Molecular Imaging Annual Meeting, Boston, MA (August 26-27, 2002).

CONCLUSIONS

In accordance with our stated goals in the first year of this project, we have successfully demonstrated the potential to image the expression of MMG in MMG-positive tumors. We plan to validate these studies and proceed with the other stated goals of the project.

REFERENCES

1. S. Achilefu *et al.*, *Journal of Medicinal Chemistry* **45**, 2003-2015 (2002).
2. M. A. Watson, T. P. Fleming, *Cancer Research* **56**, 860-865 (1996).
3. T. P. Fleming, M. A. Watson, *Ann N Y Acad Sci* **923**, 78-89. (2000).
4. M. A. Watson *et al.*, *Cancer Research* **59**, 3028-3031 (1999).
5. S. Achilefu, R. B. Dorshow, J. E. Bugaj, R. Rajagopalan, *Invest Radiol* **35**, 479-85. (2000).
6. J. E. Bugaj, S. Achilefu, R. B. Dorshow, R. Rajagopalan, *J Biomed Opt* **6**, 122-33. (2001).

APPENDICES

None