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PRINCIPAL INVESTIGATOR: Samuel Achilefu, Ph.D.

CONTRACTING ORGANIZATION: Washington University
St. Louis, MO 63110

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14. ABSTRACT

Mammaglobin (MMG) is a breast-specific glycoprotein that is over-expressed in nearly 80% of primary and metastatic breast cancers. The exact biological function of this secreted protein is not known. An important step towards understanding the role of MMG in breast carcinogenesis is to monitor its expression in MMG-expressing tumors. Toward these goals, we prepared 4 truncated MMG peptide analogues, seven MMG-avid peptides, native MMG, and anti-MMG antibodies. These molecules were labeled with near infrared and radioactive probes and evaluated in vivo and in vitro. In vitro assays show that one of these compounds are not cytotoxic, do not induce cell proliferation, and internalized i cells. The in vivo fluorescence imaging and positron emission tomography show similar biodistribution of the probes in mice. Two of the MMG-avid peptides were retained in MMG-positive tumors at higher levels relative to other compounds. These data suggest the feasibility of targeting MMG or its putative receptors for early detection of breast cancer.

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INTRODUCTION

Mammaglobin (MMG) expression is uniquely associated with human breast and, more importantly, it is up-regulated in breast cancer. Therefore, targeting MMG expression in vivo provides an opportunity to develop a novel approach to image breast cancer. 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 breast cancer and possible metastasis. This will require the use of highly sensitive methods that can detect low levels of diagnostic protein expressions in vivo. This criterion can be satisfied by the use of optical or nuclear imaging method. Therefore, this project focuses on preparing anti-MMG antibodies, native glycosylated MMG protein, and 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. We have successfully prepared novel molecular probes that are directed to MMG and anti MMG antibodies. All the MMG-related peptides and antibodies prepared are non-cytotoxic at the low micromolar concentration range that is useful for in vivo imaging. Preliminary data suggest that targeting the local concentration of MMG in breast cancer is a more useful strategy to image MMG expression than directing the probes to putative MMG receptors. The results of the project, excluding additional studies in progress, were presented at the Era of Hope meeting in Philadelphia, 2005.

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. Integrating most components of the project has enabled us to channel our resources to potentially high impact aspects of the study. The following activities were approved for this project:

TASK 1

Synthesis and labeling studies (Months 1-30)

We have successfully completed the synthesis of all the compounds proposed in the Award. We are currently scaling up the synthesis of representative compounds that preferentially localize in tumors to further explore their in vivo selectivity for breast cancer tissue.

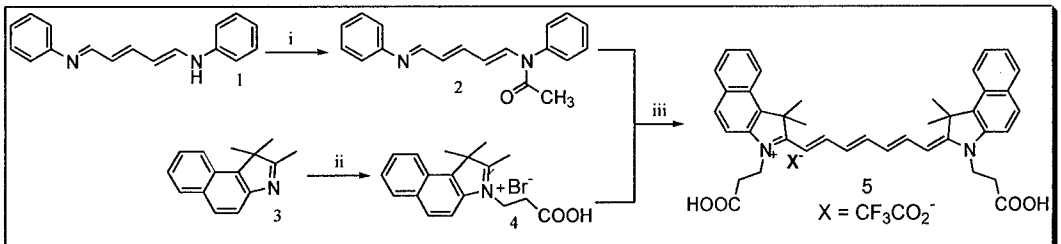
TASK 1(a)

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

We successful synthesized the optical probes needed for this study with improved yields. Part of this study is now published in the American Chemical Society Journal *Organic Letters,*[1] the Proceedings of the SPIE.,[2] *Current Medicinal Chemistry,*[3] *Technology in Cancer Research & Treatment,*[4] and *Optics Express.*[5] Two additional manuscripts are in preparation.

(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.[6] 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 a λ_{max} at about 840 nm.

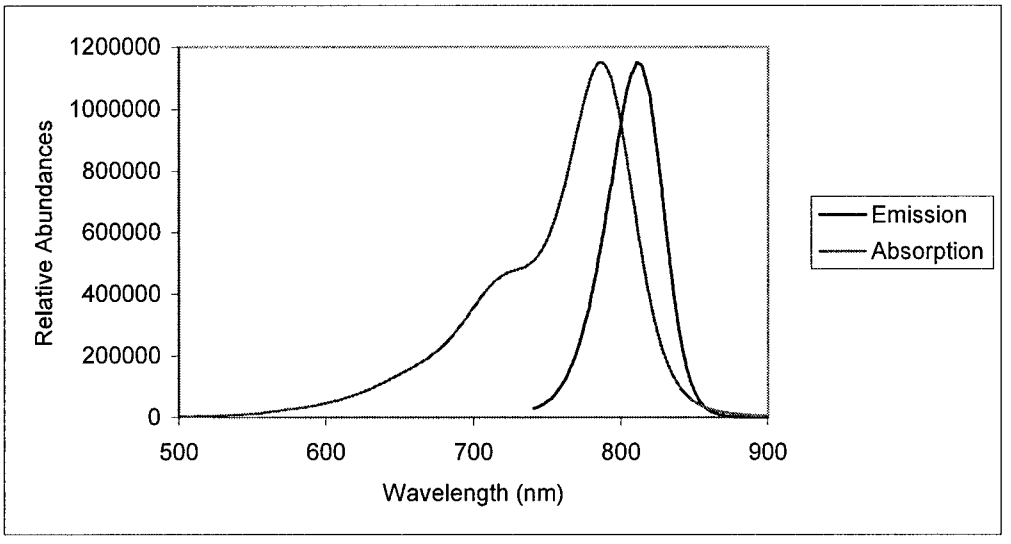


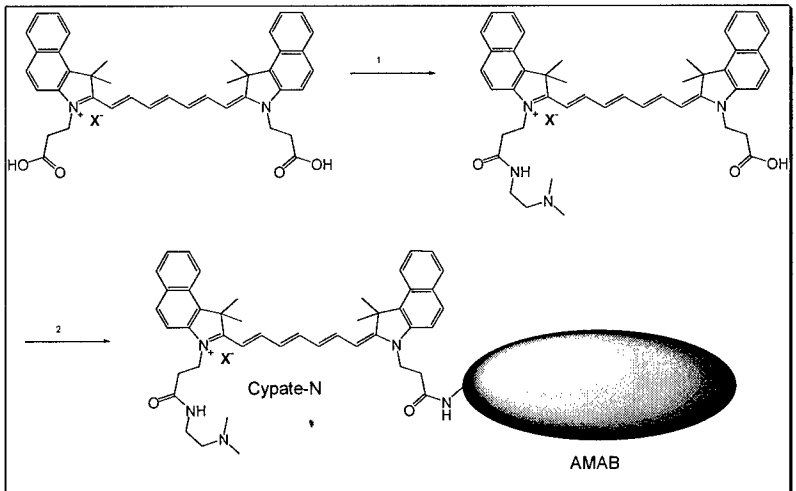
Figure 1: Absorption and Emission properties of cypate

(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. 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.

Labeling of the anti MMG antibodies with radioactive ^{64}Cu was successful and the compounds were used for in vivo biodistribution studies. However, reproducing the labeling under similar conditions without loss of immunoreactivity has been difficult. We will continue to explore alternative procedures beyond the life-span of this project. The successful procedure used are summarized below:

(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-dimethylamino propyl) 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 un-reacted 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; label with optical and radioactive probes.

(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 us-

ing enterokinase. Our carboxy terminal peptide antibody recognizes the bacterially expressed MMG protein.

(ii) **Preparation of non-glycosylated synthetic MMG:** We successfully prepared four truncated MMG derivatives by designing the fragmentation pattern to take into account the presence of cysteine residues that can cross-link the peptides by intermolecular disulfide oxidation. The following structures became the target of our new strategy to prepare MMG peptide fragments:

MMG1: **MKLLMVLMLAALSQHCYA**GSG
MMG2: **CPLLENVISKTINPQVSK**
MMG3: **TEYKELLQEFIDDNATTNA**
MMG4: **IDELKECFLNQTDETLSNVEVFMQLIYDSSLCDLF**

The cysteine residue in MMG1 was replaced with ethyl-protected cysteine amino acid [Cys(Et)-OH] to avoid the liberation of free thiol group that can crosslink with other moieties. Because conjugation of near infrared probe or DOTA will occur at the amino terminus of the peptides, no additional protection of the cysteine residue in MMG2 was needed. The steric hindrance provided by cypate or DOTA chelating group will minimize deleterious effects of the free thiol group. MMG3 has no cysteine amino acid residue and it is expected to retain the functional status of this MMG motif. MMG4 was designed to have two cysteine amino acid residues in the same fragment so that we can induce intramolecular cyclization to prevent polymerization due to cross-linkages. We used a method we reported previously in the literature to accomplish this particular synthesis.[6,7]

Procedure: The first amino acid was loaded on the resin at < 0.4 mmol/g. The peptides were prepared with ACT APEX 396 peptide synthesizer by standard Fmoc protocol,[8] as described previously.[6,9] A Wang resin pre-loaded with the first amino acid on a 30-μmole scale was placed in a reaction vessel. Subsequent Fmoc-protected amino acids were sequentially coupled to the resin-bound amino

acid from the carboxyl to amino terminus. Fmoc removal was performed with 20% piperidine in dimethyl formamide (DMF). The coupling reagents N-hydroxybenzotriazole (HOBt) and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluoro phosphate (HBTU) were used for the peptide synthesis. Peptide cleavage and concomitant removal of the amino acid side chain protecting groups were accomplished with TFA (85%), distilled water (5%), phenol (5%), and thioanisole (5%). The crude peptide was precipitated in cold *t*-butyl methyl ether (MTBE) and lyophilized in acetonitrile/water (2:3) mixture. The resulting powder was purified by HPLC and analyzed by mass spectrometry and analytical HPLC.

Conjugation of near infrared probe (cypate) or chelating group (DOTA) with the peptides: We successfully labeled the MMG peptide fragments with the metal chelating group (DOTA) and with the near infrared probe (cypate) by solid phase synthesis. This approach enabled us to obtain the desired compounds in high purity (>95%, HPLC) for in vitro and in vivo studies. Typically, the peptide was synthesized on solid support as described above and the N-terminal Fmoc was removed with 20% piperidine in DMF. Cypate or tri-*t*-butyl DOTA was pre-activated with diisopropyl carbodiimide (DIC) and added to the peptide on solid support. After 8 h, the peptide conjugate was cleaved from the resin, as described above, and the product was purified by HPLC.

Thus, we have successfully prepared MMG peptide fragments that are useful for throughput screening of putative MMG receptor in MMG-producing breast tumors.

TASK 1(d)

Identify the peptide motifs that bind to MMG.

We used five rounds of phage display reiterations to thoroughly screen and identify peptides that bind to MMG. The following peptide sequences were consistently identified by this method:

MMG 5	GAPNKAHD
MMG6	VGLVAK
MMG7	LQRGR
MMG8	GLSRAWEVDF
MMG9	RGRLQ
MMG10	MHQSG
MMG11	VTPFA

The C-terminal amide peptides shown above were prepared and labeled with Cypate and evaluated in MMG-positive breast cells. Results of the NIR fluorescent microscopy study show that the cellular uptake was highest with MMG10, suggesting that this peptide is suitable for assessing the expression of MMG in vivo. This will constitute part of future studies under a different funding support.

Figure 2: Internalization of MMG10 in cells

TASK 1(e)

Prepare and identify the AMAB peptide sequence in each antigen-binding site and synthesize MMG peptide mimics.

Monoclonal antibodies have been developed to the MMG protein[10] and we are using these reagents for our detection experiments. These monoclonal antibodies recognize the native MMG protein complex and have been used to develop an ELISA assay to detect MMG in sera and tissues. This information provides supporting data that the labeled MMG antibodies will be ideal tools for the optical detection of MMG positive breast cancer cells.

Our in vivo biodistribution studies in MD 361 tumor bearing mice identified MMG2 and MMG3 (especially MMG2) as good candidates to optimize the binding of MMG to its putative receptors.

TASK 1(f)

Optimize MMG binding affinity by semi-combinatorial approach and identify four peptides with high MMG binding affinity and specificity.

We have identified and synthesized seven different peptides that bind MMG (see Task 1d above). Using these as templates, we scaled up their synthesis and labeled all compounds with cypate for biological screening.

TASK 1(g)

Label the identified four MMG-avid peptides with a near infrared optical probe and radioactive metal chelates; purify and characterize the final compounds.

We prepared four MMG-avid peptides and showed that MMG2 and MMG3 are retained in MMG-positive breast tumor cells (see Task 1c(ii) and 1f above).

TASK 2

In vitro biological assays of mammaglobin-specific contrast agents (Months 6-30).

We used MTT assay to evaluate the proliferative and cytotoxic effects of MMG and its analogues. Cells incubated with MMG began to show signs of cytotoxicity in the micromolar concentration range while the truncated MMG peptide analogue MMG2 is not cytotoxic and do not induce cell proliferation. However, the MTT assay suggests that MMG 3 induces concentration-independent cell prolif-

eration. We also constructed a novel hybrid of MMG with the cell internalizing peptide, Tat-peptide. Western blots were used to evaluate the affinity of peptides towards MMG on MD 361 cells. The ensemble of these results demonstrates the feasibility of developing contrast agents for MMG detection. Additionally, we tested the cell proliferation and cytotoxic effects of MMG by the CyQuant assay method.

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.

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, [11-13] 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 3 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 4, 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 4, Lane 3).

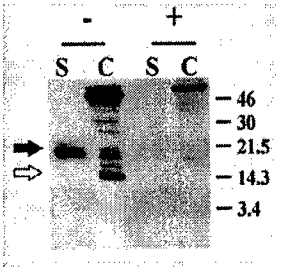


Figure 3. Detection of mammaglobin protein in the culture supernatant of the human breast cell line MDA-MB-415. Under reducing conditions, a ~21 kD band is detected by Western blot analysis in the culture supernatant (S) and a ~14 kD precursor form is observed in the cell lysate

(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

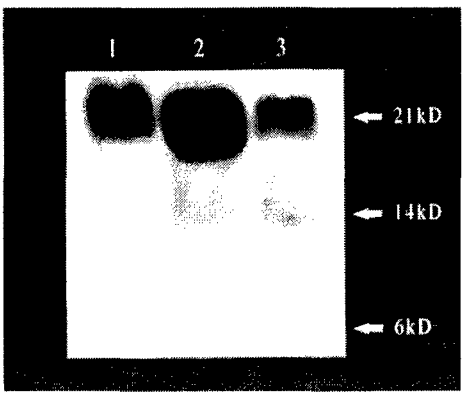


Figure 4: Treatment of native mammaglobin with N-Glycosidase F. Culture supernatants from the breast cancer cell line, MDA-MB-415were treated with no enzyme (lane 1); 15 minutes with N-Glycosidase F (lane 2); or 30 minutes treatment (lane 3). This Western blot was run under reducing conditions.

We also established that CA20948 and AR42-J cells are MMG-negative. These results enabled us to choose MDA-MB-361 and CA20948 for positive and negative controls in our studies. Unfortunately, the MDA-MB-361 and MDA-MB-415 grow very slowly in nude mice. Switching from male to female nude mice and subsequent implantation of the tumor cells in the mammary pad of female nude mice did not enhance tumor development. This problem has limited the progress of our study. Therefore, we transfected a breast tumor cell line, ZR-75-1 with MMG plasmid. This cell line is both estrogen and progesterone receptor-positive cell line used for in vitro and animal studies.[14-16] We had planned to substitute MD-MB-361 with ZR-75-1 to accelerate our imaging studies. However, the ZR-75-1 cell line lost its ability to express MMG after two passages in cell culture. Consequently, we reverted to using the slower growing MD-MB-361 for the project.

TASK 2(b)

Label MMG-positive human breast cancer cells with Zynaxis dye and assess by fluorescence microscopy the existence of MMG breast cancer receptor and preferential retention of the heavily glycosylated MMG proteins in pathologic cells due to intimate association or strong adhesion of MMG to cancer cells.

We were unable to isolate pure glycosylated MMG protein for this study. We will continue this endeavor even after the funding period, although it does not represent a major aspect of the project.

TASK 2(c)

Label MMG-positive human breast cancer cells with Zynaxis dye and determine by fluorescence microscopy the retention and specificity of MMG and AMAB peptide mimics in a mixture of labeled MMG-positive and unlabeled MMG-negative cells; identify possible fluorescence phase shift and polarization changes between bound and unbound MMG.

We labeled the 361 cells with Zynaxis dye, then mixed them with unlabeled A549 cells and incubated the mixed cells with Cypate-labeled MMG 2 and MMG3. Surprisingly, both cell types internalized the MMG compounds, as shown in Figure 5. The A549 cells also internalized these peptides when they were not mixed with 361 cells. Therefore, this part of the study requires additional effort to identify a different control cell line and discern the mechanism of uptake of these products.

Figure 5: (a) A549 and PKH2 green labeled 361 cells incubated with 1 μM MMG2 4h 37oC (470/520) (b) A549 and PKH2 green labeled 361 cells incubated with 1 μM MMG2 4h 37oC Cypate filter (755/845) (c) 361 cells are green, both A549 and 361 cells internalize MMG2

TASK 2(d)

Evaluate the internalization of AMAB, AMAB peptide mimics, synthetic MMG protein, and native MMG glycoprotein in breast caner cells to probe cytoplasmic MMG. If self-internalization is not feasible, investigate the use of Tat-peptides as adjuvant internalization agents.

We used NIR confocal fluorescence microscope for the internalization study. Cells were grown on LabTek microscope slides. The medium was removed and cells were incubated at 4 or 37 °C in PBS containing 1 μM cypate-mammaglobin peptide for various times up to 90 min. For competition studies, cells were pre-incubated with 1 μM unlabeled mammaglobin or octreotate for 15 min prior to the addition of 1 μM cypate-mammaglobin peptide conjugate. Cells were visualized with an Olympus system775/50 excitation, 845/55 emission. Of all the molecules, MMG2 and MMG10 showed higher fluorescence intensity in MMG-positive tumor cells than other MMG products. A representative image is shown in Figure 2. None of the compounds translocated into the cell nucleus.

Another task described in the proposal is to use the Tat insertion motif for internalization and detection of MMG-positive cells. Accordingly, we have prepared and purified a Tat-MMG construct. The fusion protein was tested in dendritic cells as a method of antigen delivery for T-cell activation. This preliminary study indicates the feasibility of using Tat as an "insertion" motif to internalize MMG in cells.

TASK 2(e)

Evaluate the effect of purified native MMG glycoprotein and synthetic MMG proteins on the proliferation of breast cancer cells in vitro.

Cell proliferation and cytotoxicity assays were performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based in vitro toxicology assay kit (Sigma) per the manufacturer's protocol. Briefly, MDA-MB-361

cells were grown in 96 well plates to 75% confluence in phenol red-free Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, 100 units/mL Penicillin G, 0.25 μg/mL Amphotericin B and 100 units/mL Streptomycin. The compound to be tested (0 – 125 μM) was added to each well in a final volume of 100 μL/well and the cells were incubated for 24 h at 37 °C in 5% CO_2. After incubation, the media was removed and replaced with 100 μL/well phenol red-free medium. Absorbance at 570 nm and 690 nm was measured. Ten microliters of MTT was added to each well and the cells were allowed to incubate for 2 h. The MTT solubilzation solution (100 μL of Formazan crystals dissolves in 0.04 N HCl in 2-propanol) was added to each well and formazan crystals were dissolved by vigorous pipetting. Absorbance at 570 nm and 690 nm was measured. The percent of viable cells *B* was determined according to the formula

$$B = \frac{(\lambda_{570} - \lambda_{690})_{treated}}{(\lambda_{570} - \lambda_{690})_{untreated}} \times 100,$$

where λ_{570} is the absorbance of formazan crystals at 570 nm and λ_{690} is background.

We first performed the assay with ethanol, which is an established cytotoxic agent to assure that the assay is working properly. As shown in Figure 1, nearly all the cells were no longer viable at about 30 min post-incubation. By using the same assay, we observed that native MMG was not cytotoxic up to 4 h post incubation. At 24 h, a linear decrease in cell viability was observed (Figure 6). However, we are encouraged that MMG is not cytotoxic and does not induce cell proliferation at the sub-micromole quantity we are using for in vivo studies, even after 24 h incubation period.

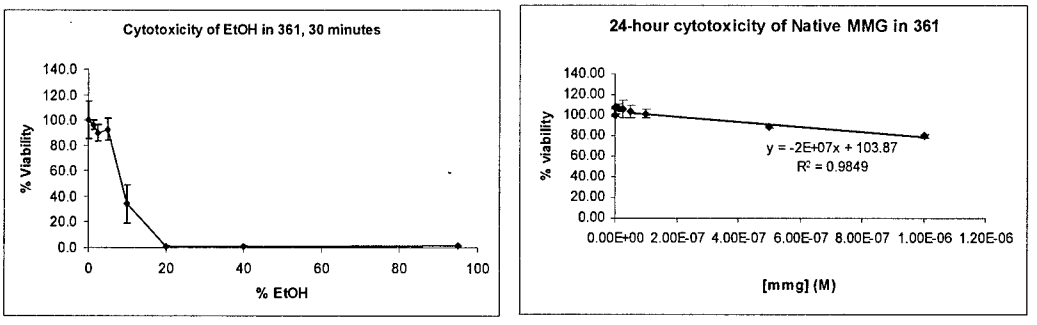


Figure 6: Cytotoxicity and cell proliferation assays for ethanol (left panel; concentration expressed as percentage of ethanol in phosphate buffer) and native MMG (right panel).

To the contrary, the synthetic peptide analogues MMG2 is apparently innocuous to cells up to 24 h at 1.0 µM and MMG3 induced cell proliferation that is not dependent on the peptide concentration (Figure 7). These data support the rationale to investigate further the development of peptide-based MMG molecular probes.

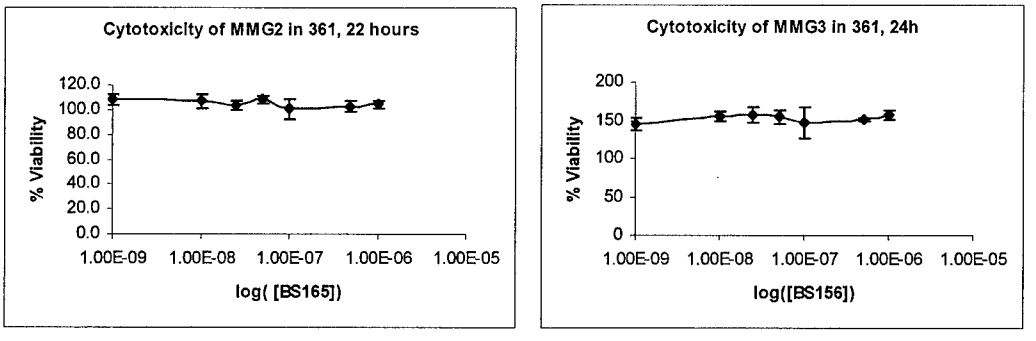
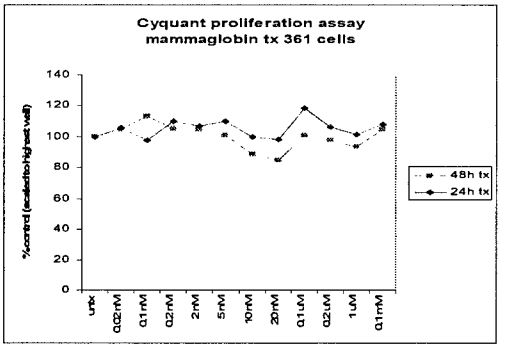


Figure 7: Cytotoxicity and cell proliferation assays for MMG2 (left panel) and native MMG (right panel).

Similarly, we evaluated the cell proliferation effect of purified native MMG by using the CyQuant proliferation assay (Molecular Probes) per the manufacturer's protocol. Cells were seeded at 2.5 x 10^4 cells/well and grown in 96 well plates in the presence of 0 – 1.5 x 10^{-5} M mammaglobin for 24 or 48 hours. Briefly, after incubation the microplate was inverted to remove the media and frozen at -70°C overnight. The microplate was thawed at room temperature and 200 µL of the CyQuant GR dye/cell-lysis buffer was added to each well. After a 5 min incubation at room temperature in the dark, the fluorescence was measured using a Synergy HT plate reader with 485 nM excitation / 520 nM emission filters. The



results show that native MMG does not have proliferative effects at a wide range of concentrations in different cell lines (Figure 8).

Fig 8 Measurement of cell proliferative effect of mammaglobin by CyQuant assay

TASK 2(f)

Develop competitive binding assays for MMG protein, AMAB and peptide mimics. Determine the binding affinity of optical and radiolabeled probes in breast cancer cell culture.

We have tested a peptide antibody, directed against the carboxy-terminal 18 amino acids of mammaglobin, and have demonstrated that this peptide antibody can bind to MMG. Importantly, the peptide can be used to successfully compete the antibody, providing a working assay for testing other peptides relevant for mammaglobin binding (Figure 9).

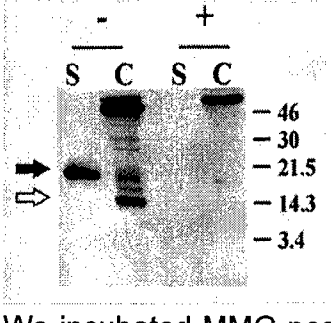


Figure 9: Detection of mammaglobin protein using a peptide antibody targeting mammaglobin. A ~21 kD band is detected by Western blot analysis in the culture supernatant (S) and a ~14 kD precursor form is observed in the cell lysate (C). The right panel (+) is identical to the left panel (-) except that competing carboxy peptide was added to demonstrate specificity of the peptide antibody generated to the mammaglobin protein. The gel was run under reducing conditions.

We incubated MMG-positive cells with MMG2 and co-incubated the peptide with unlabeled native MMG. The results indicate partial blocking of MMG2 by the peMMG, suggesting that both products may be taken up in the cells by the same receptor or process (Figure 10).

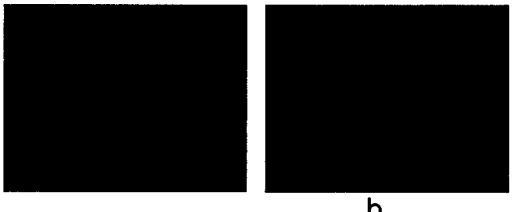


Figure 10: (a) Incubation with 1 uM MMG2 and (b) co-incubation with unlabeled MMG

TASK 3

Biodistribution and imaging of MMG-positive and MMG-negative tumor xenografts in rodents (Months 18-36).

Despite the limitations of our current breast cancer tumor model (see Task 2a), we have continued to evaluate some of the molecular probes in tumor bearing and normal nude mice. We successfully imaged the distribution of MMG products in MMG-positive tumor bearing mice by optical methods but had limited success by PET method. We used a time release estrogen pellets to facilitate tumor growth. This pellet was implanted subcutaneously in mice. We found, for the first time that this common practice in oncological research can skew observed results because the pellet retains a large amount of the injected product. Consequently, most of the in vivo experiments were performed without the estrogen pellet procedure.

(i) **Optical Imaging of cypate-AMAB and MMG derivatives 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.[9] 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 conjugates. 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.

Figure 11 shows that the fluorescently labeled anti-MMG polyclonal antibody (AMAB) was retained in the tumor and the kidneys, relative to other organs. The ex-vivo image (Figure 12) of selected organ parts confirms the whole-body optical image. The results are 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.

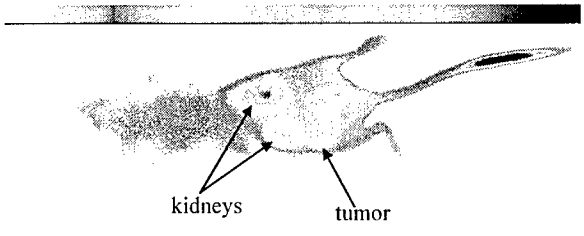


Figure 11: Cypate-AMAB conjugate injected in MDA-MB-361 tumor in nude mouse 20 hour post administration

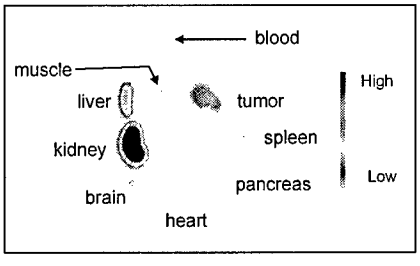


Figure 12: Ex vivo tissues at 20 hours post administration Cypate derivative/polyclonal antibody in mda-mb-361 tumor in nude mouse

Figure 13 shows that NIR fluorescently labeled MMG2 was retained in the tumor at 5 h postinjection. At 24 h, however, the probe completely cleared from blood and localized predominantly in the kidneys (ex-vivo image).

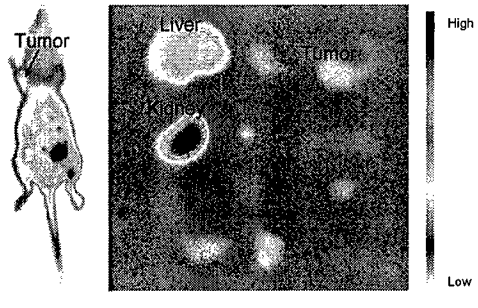


Figure 13: Distribution of Cypate MMG2 in a MDA-MB-361 tumor-bearing nude mouse. Whole body image (left panel) is at 5 h postinjection and organ parts (right panel) image was taken at 24 h postinjection.

Using the statistical package in WinView, we determined the relative accumulation of the probe in ex-vivo organ parts (Figure 14). The result shows that the selectivity of the probe for the tumor is not high relative to other organs. This could result from the small size of the tumor used, which was hardly palpable, or the probe may have low affinity for putative receptor on the surface of the tumor cells. It is also possible that MMG receptors are not present on the tumor surface. Alternatively, when compared with the good image obtained with the polyclonal antibody labeled near infrared probe, the result may indicate that the best approach to image the expression of MMG is to target its local concentration in the vicinity of the tumors by using anti-MMG antibody.

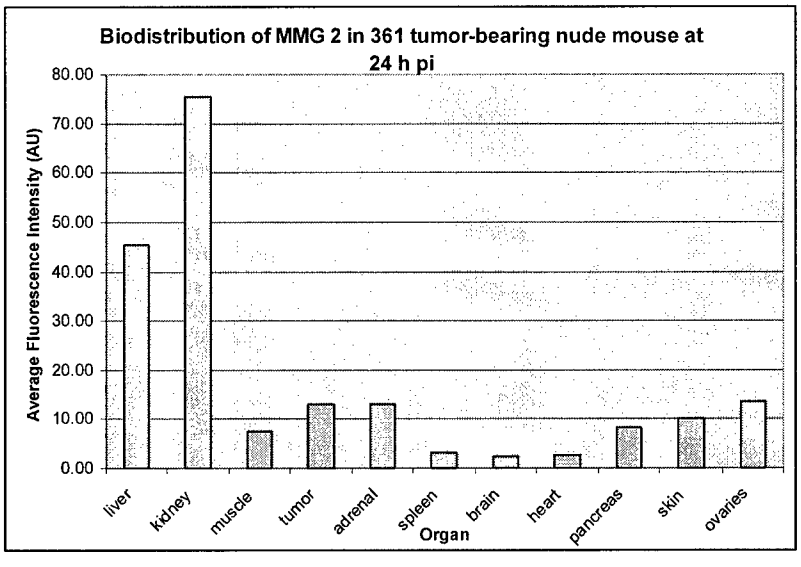


Figure 14: Ex-vivo distribution cypate MMG2 in MDA-MB-361 tumor-bearing nude mouse tissues at 24 h postinjection.

We were surprised to observe that excess unlabeled MMG did not block the update of MMG2 in mice, as shown in Figure 15. This could suggest a higher binding affinity of the new molecular construct towards the putative MMG receptor or that both compounds bind to different sites of the receptor. We will be applying for NIH funding to explore these and other possibilities further.

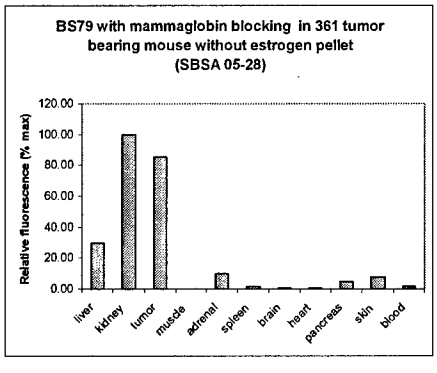


Figure 15: MMG3 and mammaglobin co-injected in 361 tumor bearing mouse 24 h post injection

A similar result described above was obtained with MMG3, except that this compound equally distributed in the liver and the kidneys (Figure 16). The tumor uptake was modest.

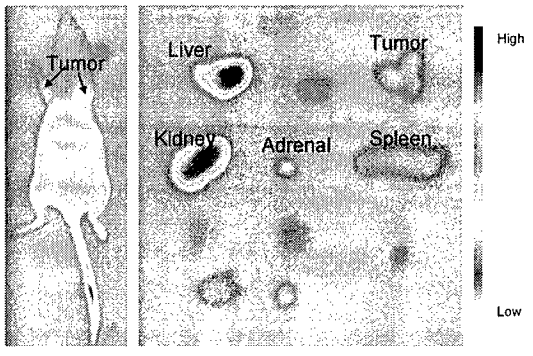
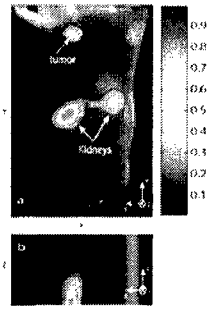


Figure 16: Distribution of Cypate MMG3 in a MDA-MB-361 tumor-bearing nude mouse. Whole body (left panel) and organ parts (right panel) images were taken at 24 h postinjection.



Recently, we developed a new fast-scanning diffuse optical tomography system and used it to image the distribution of MMG3 in MMG-positive nude mice (Figure 17).[5]

Fig. 17. Representative slices from a 3D tomographic reconstruction of a nude mouse with a subcutaneous breast-specific human breast cancer xenograft MDA MD 361 after administration of MMG2. a) a xy slice parallel to the detector plane at a depth of z = 12mm and b) a xz slice extending from the source plane to detector plane at y = 15mm .

(ii) Radiolabeling of DOTA-MMG-Peptide and AMAB with ^{64}Cu and microPET imaging in MDA-MB-361 tumor-bearing mice

(a) Radiolabeling: The labeling of MMG2 peptide analogue is representative of the method used to prepare the radiopharmaceuticals for PET imaging studies. Conjugation of the peptide with the chelating group (DOTA) was accomplished on solid support, as described in Task 1c. The peptide was dissolved in 480 µL of 0.1M NH_4CIT buffer (pH 7.5), giving a colorless clear solution at a concentration of 0.5 µg/µL. The stock solution was stored at -80 °C. To radiolabel the peptide, DOTA-MMG2 solution (10-µg or 5-µg) was diluted with the NH_4CIT buffer to 100 µL, to which ~2.5 mCi of ^{64}Cu in 20 µL of 0.1M NH_4CIT (pH 7.5) was added. The resulting solutions were incubated at 43 °C for 30-min (1000 rpm). The reaction mixture was transferred to a C18 SepPak cartridge pre-washed with 5 mL of ethanol, followed by 5 mL of water. The SepPak was washed with 5 mL of water and the product eluted with 1 mL of ethanol. Different fractions of the ethanol

eluents were collected in three vials and characterized by HPLC equipped with UV and radioactivity detectors. Two of the vials were combined to give 264 µCi in ~ 400 µL. Quality control by HPLC showed that the DOTA-MMG2 was successfully labeled with ^{64}Cu and its radiochemical purity was nearly 100%.

(b) MicroPET imaging: MDA-MB-361 human breast cancer cells were injected into the mammary fat pad of female nude mice. The tumor growth was very slow and after >2 months, it was still difficult to obtain palpable tumor mass. The mice were used in this form for in vivo biodistribution of the probes by microPET. Two of the nude mice were injected with 100 µCi (150 µL) of the radiolabeled MMG2 peptide analogue per mouse. Images at 0.5, 1, 4, and 24 h pi were taken.

The microPET images of ^{64}Cu-DOTA-MMG2 are shown in Figure 18. Tumor was not visible at any time points. It is probably because the tumor was too small to be imaged, or the tumor targeting specificity of ^{64}Cu-DOTA-MMG-Peptide was low. At 30 min postinjection, both mice showed a hot bladder. This indicates that some of the ^{64}Cu-DOTA-MMG2 was excreted via kidneys. The activity also cleared via intestines, as shown in the images at 30 min and 4 h postinjection.

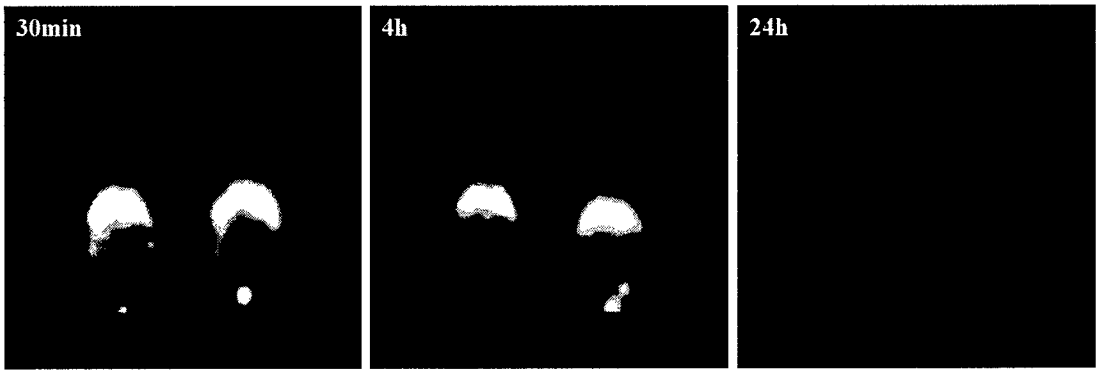


Figure 18: MicroPET coronal images of ^{64}Cu-DOTA-MMG2 in MC7-MAM tumor-bearing mice (n = 2). The image intensity is at the same scale.

The time-activity curves in visible organs are shown in Figure 19. It confirmed the observation that a significant amount of ^{64}Cu-DOTA-MMG2 was cleared via kidneys, which correlates with results of the optical imaging. Subsequently ex-vivo

evaluation did not reveal the presence of the MMG-positive tumor in mice used for this study.

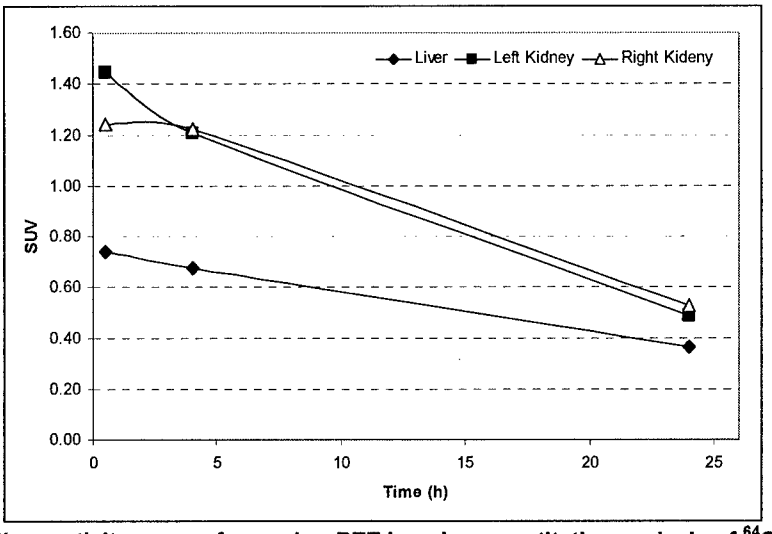


Figure 19: The time-activity curves from microPET imaging quantitative analysis of ^{64}Cu-DOTA-MMG-Peptide in MC7-MAM tumor-bearing mice.

A similar procedure for labeling and imaging MMG2 peptide analogue was used to evaluate the anti MMG monoclonal antibody developed for this study, except that the DOTA was first conjugated to the antibody by standard succinimidyl ester activation mechanism. The microPET images of ^{64}Cu-DOTA-AMAB are shown in Figure 20.

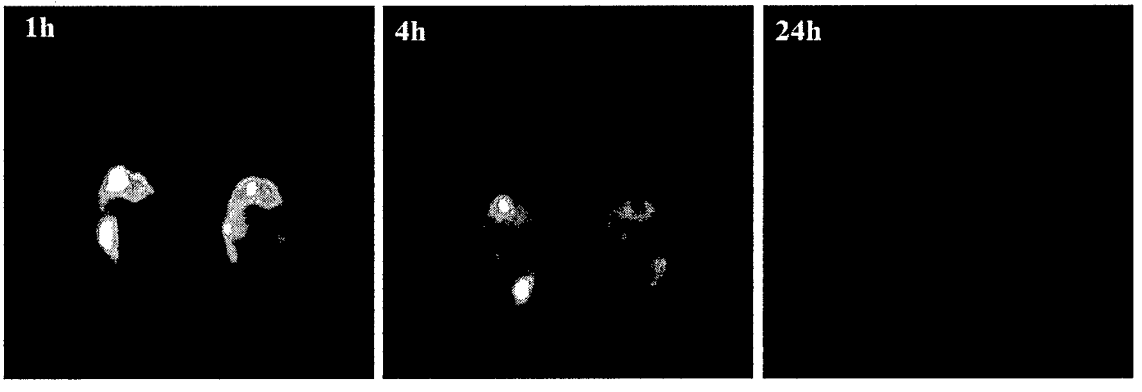


Figure 20: MicroPET coronal images of ^{64}Cu-DOTA-AMAB in MDA-MB-361 tumor-bearing mice (n = 2). The image intensity is on the same scale.

The time-activity curves in visible organs are shown in Figure 21. It appears that ^{64}Cu-DOTA-IgG was cleared via intestines.

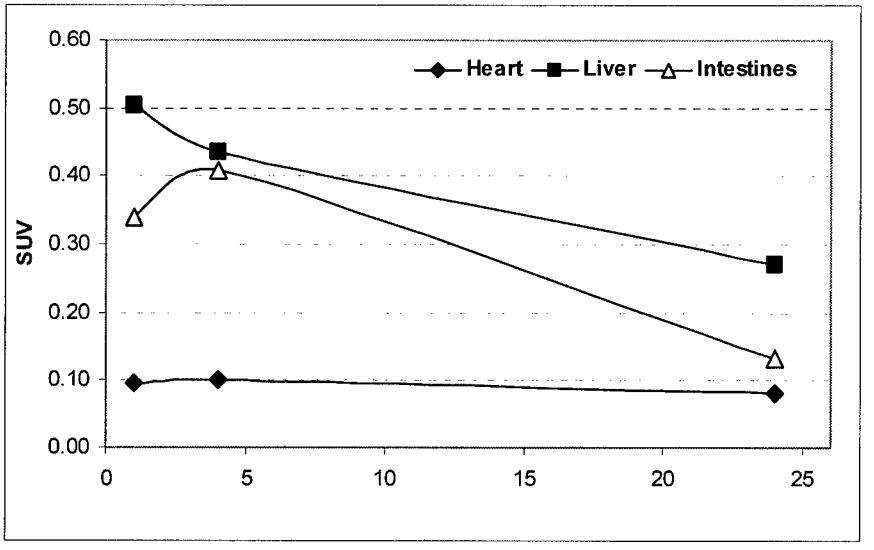


Figure 21: The time-activity curves from microPET imaging quantitative analysis of ^{64}Cu-DOTA-IgG in MDA-MB-361 tumor-bearing mice.

We have had difficulties with reproducing the labeling of native MMG and the AMAB without loss of immunoreactivity. A new faculty with expertise in protein labeling chemistry has been hired ans he is working on optimizing the labeling conditions. When these studies are completed, the product will be administered in breast cancer tumor bearing mice for PET studies. All future publications emanating from these studies will acknowledge the financial support of this DoD grant.

KEY RESEARCH ACCOMPLISHMENTS (2001-2005)

(1) We have prepared the first series of imaging agents for monitoring MMG expression by optical and nuclear methods.

(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.

(4) In vitro studies indicate that MMG may have anti-proliferative effect on breast cancer cells at > 1 μM concentrations but does not induce proliferative effects on cancer cell. Interestingly, contrasting results were obtained with the MMG peptide fragments. While MMG3 appears to induce a concentration-independent proliferative effect, MMG2 was found to be neither cytotoxic nor induce cell proliferation.

(5) A Tat-MMG fusion protein has been developed.

(6) Optical imaging data is ambiguous concerning the presence of a putative cell surface receptor for MMG. A preferred strategy to image MMG expression and, hence, detect breast cancers, is to develop and label molecules that bind to MMG with radionuclear and optical probes.

(7) A fast scanning diffuse optical tomography system has been developed and used to obtain high resolution images of probe localization in breast cancer xenograft in mice.

REPORTABLE OUTCOMES

Presentations:

Invited Presentations:

1. S. Achilefu: Rational design, synthesis, and biodistribution of disease-specific molecular probes. 4th Annual Meeting of the Society for Molecular Imaging, Cologne, Germany (September 7-10, 2005)

2. S. Achilefu: Molecular imaging 101 - Optical imaging primer. 52nd Annual Meeting of the Society for Nuclear Medicine, Toronto, Canada (June 20, 2005)

3. S. Achilefu: Harnessing the power of light to non-invasively image and localize diseases in small animals. GE Asia-Pacific World Lecture Series – S. Korea: Busan; China: Shanghai and Beijing; New Zealand: Auckland; Australia: Sidney and Melbourne; and Thailand: Bangkok; (May 9-20, 2005)

4. S. Achilefu: Highly specific optical molecular probes for imaging tumors. Health Science and Engineering Seminar Series, University of Michigan, Ann Arbor, MI (March 15, 2005)

5. S. Achilefu: Applications of optical molecular imaging in biology and medicine. Molecular Imaging Workshop, San Jose, CA (January 23, 2005)

6. S. Achilefu: Molecular imaging. Guest Speaker (Hot Topics) at the Biomedical Optics Society Symposium, SPIE Photonics West, San Jose, CA (January 22, 2005)

7. S. Achilefu: Highly specific optical molecular probes for imaging tumors and monitoring tumor therapy. *Frontiers in Optics 2004*, Optical Society of America, Rochester, NY (October 10-14, 2004)

8. S. Achilefu: Molecular designs for targeting and imaging human diseases in small animal models by optical. University of Wisconsin Biomedical Engineering Seminar Series, Madison, WI (October 4, 2004)

9. S. Achilefu: Recent advances in optical molecular probes. Optical Imaging Workshop 2004: Fourth Inter-Institute Workshop on Optical Diagnostic Imaging from Bench to Bedside at the NIH, Bethesda, MD (September 20-22, 2004)

10. S. Achilefu, Y. Ye, S. Bloch, Z. Zhang, M. Berezin, K. Liang. Development of Contrast Effectors for Optical and Multimodal Imaging of Tumors. 228th American Chemical Society National Meeting, Philadelphia, PA (August 22-26, 2004)

11. S. Achilefu, Y. Ye, S. Bloch, Z. Zhang, R. Dorshow, K. Liang, Molecular Optical Probes for Imaging Tumors. *IEEE/LEOS* Summer Topical Meeting, San Diego, CA (June 28-30, 2004)

12. S. Achilefu: The role of optical molecular probes in tumor imaging and organ function monitoring. Seminar Series, Intramural Research, National Institute of Child Health & Human Development, NIH, Bethesda, MD (December 8, 2003)

13. S. Achilefu: Lighting up tumors by optical methods. Roswell Cancer Institute Seminar, Buffalo, NY (December 1, 2003)

14. S. Achilefu: Contrast Agent-Mediated Optical Imaging of Tumors. Fall Meeting, American Association of Physicists in Medicine, Missouri River Valley Chapter, St. Louis, MO (November 8, 2003)

15. S. Achilefu: Receptor-specific near infrared molecular probes for imaging aberrant protein expression in vivo. *MGH Wellman Laboratories of Photomedicine Lecture Series*, Boston, MA (September 30, 2003)

16. S. Achilefu, Y. Ye, S. Bloch, R. Dorshow, K. Liang, T. Fleming: Near infrared molecular probes for imaging protein expression. *Imaging in 2020,* Jackson Hole, Wy (September 7-11, 2003)

17. S. Achilefu: Contrast agent-mediated organ function monitoring and tumor imaging by optical methods. *Advances in Optics for Biotechnology, Medicine and Surgery*, Banff, Alberta, Canada (August 3-7, 2003)

18. S. Achilefu, Y. Ye, S. Bloch: Diagnosis and treatment of tumors by optical methods. *The 31st Annual Meeting of the American Society for Photobiology*, Baltimore, MD (July 5-9, 2003)

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

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

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

22. S. Achilefu: Optical contrast agent-mediated imaging of tumors. *Advanced Research Technologies Seminar Series*, Montreal, Canada (October 16, 2002)

23. S. Achilefu: Optical contrast agents for tumor imaging. *NIH Workshop on Optical Imaging*, Bethesda, MD (September 26-27, 2002)

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

Other Presentations:

25. T. Fleming, S. Bloch, K. Liang, K. Block, M. Hartman, X. Sun, M. Welch, C. Anderson, S. Achilefu: Optical imaging of mammaglobin expression. 4th Era of Hope Meeting, DOD Breast Cancer Research Program Meeting, Philadelphia, PA (June 8–11, 2005; poster presentation).

26. Z. Zhang, S. Achilefu: Synthesis and evaluation of polyhydroxylated near-infrared carbocyanine molecular probes. *International Symposium on Biomedical Optics*, SPIE, San Jose, CA (January 24-29, 2004).

Publications:

(1) Zhang, Z.; Achilefu, S. Synthesis and evaluation of polyhydroxylated near-infrared carbocyanine molecular probes. *Organic Letters* **2004**, *6*, 2067-2070.

(2) Zhang, Z.; Bloch, S.; Achilefu, S. Synthesis and Evaluation of Novel Galactose-Carbocyanine Fluorescent Contrast Agents with Enhanced Hydrophilicity and Rigid Molecular Constraint. *Proc SPIE* **2004**, *5329*, 262-268.

(3) Achilefu, S. Optical Imaging Agents and Potential Application in the Assessment of Pancreatic Beta Cells. *Current Medicinal Chemistry - Immunology,Endocrine Metabolic Agents* **2004**, *4*, 253-269.

(4) Achilefu, S. Lighting up tumors with receptor-specific optical molecular probes. *Technology in Cancer Research & Treatment* **2004**, *3*, 393-409.

(5) Patwardhan, S. V.; Bloch, S. R.; Achilefu, S.; Culver, J. P. Time-dependent whole-body fluorescence tomography of probe bio-distributions in mice. *Optics Express* **2005**, *13*, 2564-2577.

CONCLUSIONS

We have successfully demonstrated the potential to image the expression of MMG in MMG-positive tumors in mice. Our results indicate that labeling molecules that bind MMG is a preferred strategy to image the expression of MMG in breast cancer than targeting putative cell surface MMG receptor. While the optical imaging results convincingly demonstrated the potential to target MMG or its putative receptor for imaging breast cancer, additional studies will be required to validate this strategy. Development of MMG-specific peptide is also desirable to mitigate the problems associated with labeling antibodies. We will be seeking other sources of funds to continue this line of research.

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APPENDIX: Current Address Information

Current Address:

Samuel Achilefu, PhD
Associate Professor of Radiology
Department of Radiology
Washington University School of Medicine
Campus Box # 8225
4525 Scott Avenue - East Building
St. Louis, MO 63110-1093

Phone: 314 362 8599

Fax: 314 747 5191

E-mail: achilefus@mir.wustl.edu

Participants on the project:

(1) Samuel Achilefu, PhD*
(2) Timothy Fleming, PhD*
(3) Carolyn Anderson, PhD*
(4) Joseph Culver, PhD
(5) Sharon Bloch, PhD*
(6) Zongren Zhang, PhD*
(7) Sachin Patwardhan, PhD
(8) Xiankai Sun, PhD
(9) Mikhail Berezin, PhD
(10) Kexian Liang
(11) Laura Meyer
(12) Melissa Hartman

* Paid from grant