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Approved for Public Release; Distribution Unlimited**12b. DISTRIBUTION CODE****13. ABSTRACT (Maximum 200 Words)**

Receptors for vasoactive intestinal peptide (VIP-R) are overexpressed in human breast cancer. This phenomenon may have important diagnostic and therapeutic implications because carrier systems such as sterically stabilized liposomes (SSL) loaded with imaging or therapeutic agents, and with surface ligands specific to VIP-R could potentially be actively targeted to breast cancer. This part of the project aims to test the targeting ability of VIP-SSL to n-methyl nitrosourea (MNU)-induced rat breast cancer in vivo. Previously we conjugated VIP to an activated DSPE-PEG-NHS and the DSPE-PEG-VIP was inserted into preformed radionuclide (Technetium)-loaded SSL by incubation at 37°C. Fluorescent labeled VIP-SSL demonstrated significant binding to MNU-induced rat breast cancer tissue sections in vitro. In this part of the study we evaluated the pharmacokinetic behavior and the in vivo targetability and tissue distribution of Tc-loaded VIP-SSL in rats bearing MNU-induced breast cancer. The VIP-SSL demonstrated long circulating capability (half-life ~16h) with a monophasic decline. Significantly more accumulation of VIP-SSL was observed in the breast tumors as compared to normal breast tissue demonstrating successful passive targeting due to extravasation. Moreover VIP-SSL accumulated significantly more, compared to SSL, in breast tumors and indicated success in active targeting. This targeted carrier system is currently being explored for improved functional imaging of breast cancer.

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INTRODUCTION

The primary goal of the past year's work was to determine the in vivo behavior of the targeted sterically stabilized liposomes incorporating Tc99m which we developed in the previous year, in rats bearing carcinogen-induced breast cancer. The blood profile of VIP-SSL was determined in comparison to SSL to evaluate its circulation behavior and its longevity in the circulation. This targeted system was then evaluated for its passive and active targeting ability in vivo by determining the tumor and tissue distribution of the Tc99m-loaded SSL with and without surface VIP in rats with breast cancer.

BODY

Task 1: Develop labeled VIP-SSL (Year 1)

Preparation of SSL encapsulating Tc99m:

Sterically stabilized liposomes were prepared by hydration of dried lipid film followed by extrusion, as described before (Dagar, 1998) with modifications. Egg-phosphatidylcholine (PC), cholesterol (CH), polyethylene glycol (molecular weight 2000) conjugated distearyl phosphatidylethanolamine (DSPE-PEG) & dipalmitoyl phosphatidylglycerol (DPPG) in the molar ratio PC: DPPG: DSPE-PEG: CH of 0.50:0.10:0.05:0.35 were dissolved in an organic solvent (chloroform-methanol; 9:1 v/v) & solvent evaporated in a rotary evaporator under vacuum. The dry lipid film was hydrated with isotonic, 50 mM glutathione containing isotonic 0.01M HEPES buffer (pH 7.4). The dispersion was extruded through polycarbonate filter (100 nm) and untrapped glutathione removed by gel filtration with isotonic 0.01M HEPES buffer (pH 7.4) as the eluent. The glutathione containing liposomes, visible as turbid fractions, were pooled. These liposomes were then labeled by first incubating Ceretec[®] with Tc99m-pertechnate to form a lipophilic Tc99m-HMPAO complex. This lipophilic complex was then incubated with preformed glutathione-containing liposomes and the complex, being lipophilic, passed through the bilayer. Tc-99m-HMPAO complex was then trapped irreversibly in the internal aqueous phase of the liposome by reduction of the lipophilic complex by glutathione into a hydrophilic one. The free label was then removed by gel filtration. The Tc-99m-HMPAO encapsulating liposomes (turbid fractions with high radioactivity) coming out in the void volume were pooled and used for further studies. The mean size of the prepared liposomes and the radioactivity in each fraction were measured using QuasiElastic Light Scattering and a dose calibrator respectively.

Results: SSL with average size of ~100 nm were successfully prepared and a clear separation of the encapsulated Tc99m-HMPAO was seen as shown in Figure 1.

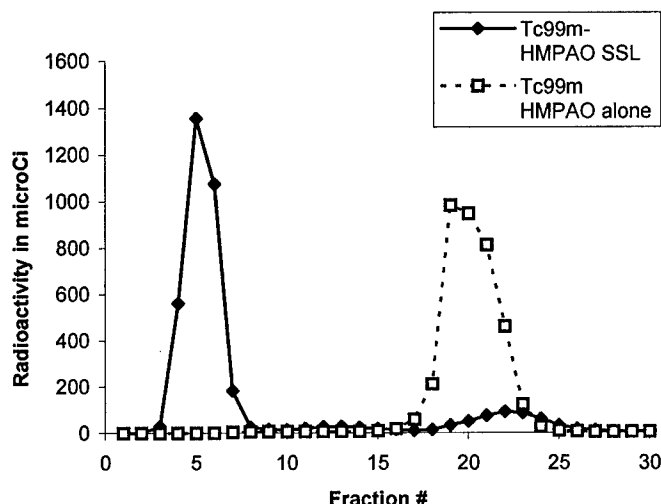


Figure 1: Elution profile of free Tc99m-HMPAO and Tc99m-HMPAO encapsulating SSL after gel filtration

Conjugation of VIP to DSPE-PEG and insertion into SSL:

An activated DSPE-PEG₃₄₀₀ (DSPE-PEG-NHS, 1,2-dioleoyl-*sn*-glycero-3- 3400 phosphoethanolamine-*n*-[poly(ethylene glycol)]-*N* hydroxy succinamide, PEG *Mw* 3400) was used to conjugate VIP to DSPE-PEG. This 3400 reaction takes place between amines and NHS group, which acts as the linking agent. VIP and DSPE-PEG-NHS in the molar ratio of 1:5 (VIP: DSPE-PEG-NHS) were dissolved separately in 3400 0.01 M isotonic HEPES buffer, pH 6.6. DSPE-PEG-NHS solution was added in small increments over 1–2 min to the VIP solution at 48°C with gentle stirring. The reaction was allowed to proceed for 2 h at 48°C and then stopped by adding glycine solution to the reaction mixture to consume the remaining NHS moieties. The conjugation was tested using SDS-PAGE and subsequent staining with first Coomassie Blue R-250 and then silver stain. The bioactivity of the conjugated VIP was tested using an in situ hamster cheek pouch bioassay. The VIP conjugated to DSPE-PEG (DSPE-PEG-VIP) was subsequently used to prepare fluorescent VIP-SSL.

The conditions for DSPE-PEG conjugated VIP insertion into preformed Tc-99m-HMPAO encapsulating SSL were determined by measuring the amount of DSPE-PEG in the preformed liposomes under various conditions and times.

Results: A 1:1 conjugate of DSPE-PEG and VIP was successfully prepared (Figure 2). In addition in situ bioassay indicated that the bioactivity of VIP was retained after conjugation. DSPE-PEG-VIP was maximally inserted at about 2h. There was no significant increase in insertion into the liposomes after 2h. Hence, 2h incubation at 37°C was considered enough to insert significant amounts of DSPE-PEG-VIP into the preformed SSL.

Characterization of Tc99m-HMPAO encapsulating VIP-SSL

The Tc99m-HMPAO encapsulating VIP-SSL were characterized in terms of their size, phospholipid and radioactivity content and their labeling efficiency and compared to Tc99m-HMPAO encapsulating SSL. The release of Tc99m-HMPAO encapsulating

SSL after insertion of conjugate was tested by storing of SSL in the presence and absence of DSPE-PEG₃₄₀₀-NHS at 37°C.

Results: There was no significant difference between Tc99m-HMPAO encapsulating VIP-SSL and SSL (Table 1) indicating that insertion of VIP did not interfere with the properties of the SSL. No significant leakage of Tc99m label (Figure 3) and change in size (Before incubation 109±13 nm and after incubation 114±15 nm) was observed, indicating that these liposomes were stable.

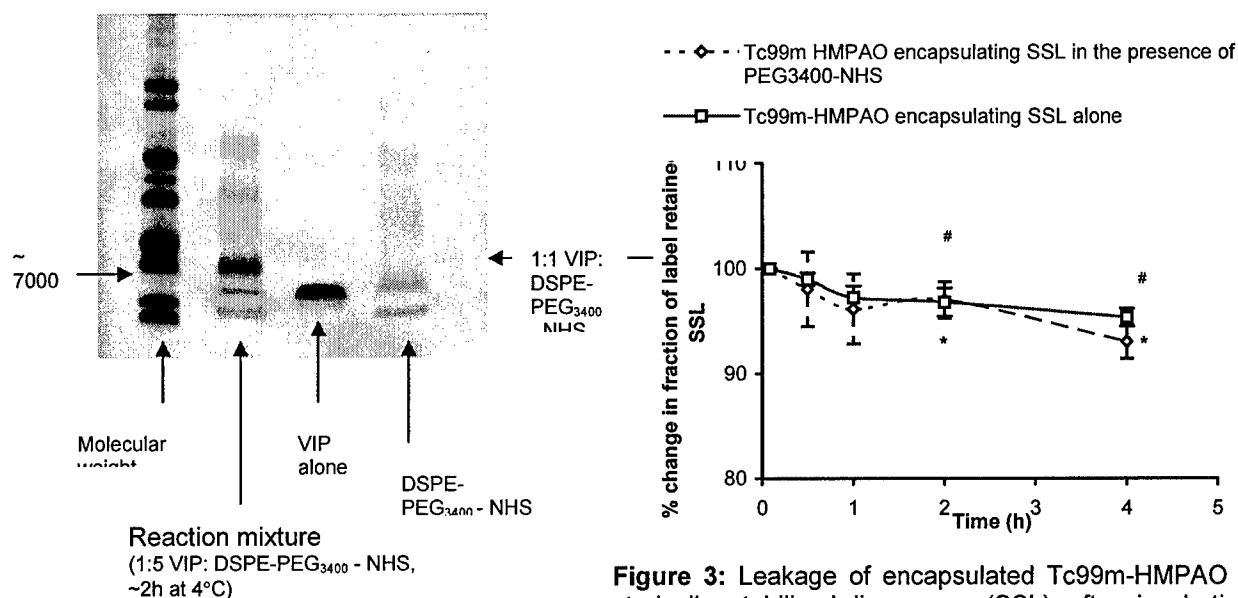


Figure 2: SDS-PAGE of the reaction products.

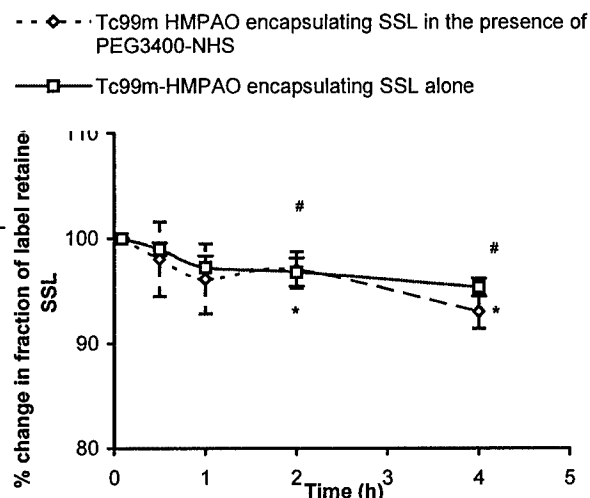


Figure 3: Leakage of encapsulated Tc99m-HMPAO from sterically stabilized liposomes (SSL) after incubation at 37°C in isotonic 0.01M HEPES buffer (pH 7.4).

Table 1: Characteristics of Tc99m-HMPAO encapsulating SSL and VIP-SSL (Each value is Mean ± standard deviation, n = at least 6)

CHARACTERISTIC	METHOD	Tc99m-HMPAO encapsulating SSL	Tc99m-HMPAO encapsulating VIP-SSL
SIZE	Qausi - elastic light scattering	109.81 ± 14.21 nm	114.77 ± 13.72 nm
PHOSPHOLIPID CONTENT	Modified Bartlet Phosphate assay	3.15 ± 0.23 µmol/mL	3.01 ± 0.48 µmol/mL
RADIOACTIVITY CONTENT	Atomlab 100 dose Calibrator	1008 ± 160 µCi/mL	800 ± 113 µCi/mL
LABELING EFFICIENCY	Atomlab 100 dose Calibrator	85.7 ± 4.46 %	83.8 ± 2.42 %

Status of Task 1: COMPLETED (Year 1)

Task 2: Test the *in vitro* Targeting of labeled VIP-SSL to VIP-R (Year 1)

For testing the *in vitro* binding, BODIPY-Chol (a non-exchangeable fluorescent probe) containing liposomes, were prepared using film rehydration-extrusion method, as described above but incorporating the probe at 1:1500 molar ratio (lipid:probe) in the lipid mixture. DSPE-PEG-VIP was inserted into these fluorescent liposomes to form fluorescent VIP conjugated sterically stabilized liposomes (VIP-SSL).

The rats bearing MNU-induced breast cancer were developed as described below and were euthanized by exposure to carbon dioxide in a closed chamber. Normal and cancerous breast tissues were excised, frozen immediately in liquid nitrogen and stored at -80°C until use. The frozen breast tissue was cut into 20 micron sections and mounted on microscopic slides. They were then fixed with 4% formaldehyde and allowed to air-dry for 10 min. Adjacent 5 micron thick frozen tissue sections, were stained with hematoxylin and eosin to confirm the presence or absence of cancer in the breast tissue. The presence of VIP-R in these rat breast cancer tissues was confirmed using a fluorescent VIP, Fluo-VIP as described by us recently [Dagar 1999 and 2001]. Twenty-micrometer sections of MNU-induced rat breast cancer tissues were cut using a cryotome, placed on a slide, fixed with 4% formalin for 20 min, and then air-dried for 10 min. The BODIPY-Chol containing VIP-SSL were added to the sections and incubated for 1 h at room temperature. At the end of the incubation period, the slides were washed with 0.01 M isotonic HEPES buffer, pH 7.4, four times for 60s each. The slides were then observed with a Zeiss Fluorescence microscope attached to a Zeiss Camera (Carl Zeiss Inc., Thornwood, NY) and photographed.

Results: Figure 4 shows the fluorescence microphotographs of breast cancer tissues. The microphotographs indicate that more VIP-SSL was attached to MNU-induced rat breast cancer tissue sections while SSL without VIP or with non-covalently associated VIP, showed no significant attachment. This data indicated that VIP-SSL were able to bind to breast cancer tissue *in vitro* and it was likely that same would be true *in vivo*.



Figure 4: Microphotographs of MNU-induced rat breast tumor tissue sections incubated with fluorescent liposomes A. BODIPY-Chol incorporating fluorescent VIP-SSL (with covalently attached VIP) B. BODIPY-Chol incorporating fluorescent SSL (without VIP) C. BODIPY-Chol incorporating fluorescent SSL (with non-covalently associated VIP).

Status of Task 2: COMPLETED (Year 1)

Task 3: Determine the biodistribution of labeled VIP-SSL in Breast Cancer Bearing Rats. (Year 2)

Breast cancer induction:

Breast cancer was induced in rats with MNU as previously described [Dagar 1998]. Briefly, virgin female Sprague–Dawley rats, 36 days old, weighing ~140 g, were anesthetized with ketamine/ xylazine (13.3 / 1.3 mg per 100 g body weight, i.m.). Each animal received a single intravenous injection of MNU (50 mg/kg body weight) in acidified saline (pH 5.0), via the tail vein. The rats were weighed weekly. They were palpated every week, starting at 3 weeks post-MNU administration. Palpable mammary tumors were detected within 100–150 days after injection. When tumors were ~2cm in diameter they were utilized for the in vivo studies.

Biodistribution studies:

In order to determine the targeting ability of the Tc loaded-VIP-SSL to the breast tumors biodistribution studies were performed on the tumor bearing rats. Rats with induced mammary tumors were divided into two groups of five rats. Each group was injected with 300 μ Ci as Tc-labeled-VIP-SSL or Tc-labeled-SSL by the tail vein. At 27 h post-injection, the rats were euthanized by an over- dose of ketamine/ xylazine. A blood sample was obtained by cardiac puncture. Tissues (normal breast or breast cancer, liver, spleen, kidneys, calf muscle, heart and lungs) were dissected, washed with saline, dried and transferred to pre-weighed polypropylene tubes and weighed. Their activity was measured using a scintillation gamma counter (Cobra 5005, Packard Instruments). To correct for the physical decay of Tc99m, a 10 ml aliquot of the injected dose was also counted. The uptake was measured and expressed as percent injected dose per gram of tissue muscles (% I.D. /g). The tissue radioactivity amounts, % I.D. /g, were compared between formulations (Tc-99m-HMPAO encapsulating SSL or Tc-99m-HMPAO encapsulating VIP-SSL) as well as between normal and tumor bearing rats for each of the formulations using unpaired Student's *t*-test. $P < 0.05$ was considered significant.

Results: The tissue and tumor distribution of SSL and VIP-SSL in healthy and tumor bearing rats are shown in tables 2 & 3 and Figure 5.

The normal breast uptake in healthy rats for both Tc-99m-HMPAO encapsulated SSL and Tc-99m-HMPAO encapsulated VIP-SSL was only 0.04 % I.D. per gram of tissue (Table 2). In contrast, the breast tumor uptake for Tc-99m-HMPAO encapsulated SSL was about 3 times more than in normal tissues suggesting that the SSL were passively targeted to the breast tumor due the leaky vasculature present in the tumor and the correct size of liposomes (~100 nm). For Tc-99m-HMPAO encapsulated VIP-SSL the uptake was about 6 times more than in normal tissues and 2 times more in the tumors in comparison to SSL (Table 3). This significant increase in accumulation of Tc-99m-HMPAO encapsulated VIP-SSL in breast tumor as compared to SSL indicates success in active targeting resulting in retention of the liposomes by receptor interaction at the tumor site after extravasation leading to significant improvement in the accumulation of SSL into the breast tumor.

There was no major difference in the accumulation of Tc-99m-HMPAO encapsulated SSL and Tc-99m-HMPAO encapsulated VIP-SSL in other tissues (heart, lungs, liver, blood and muscle) on both normal and breast tumor bearing rats.

Table 2: Biodistribution of Tc99m-HMPAO encapsulating SSL and Tc99m-HMPAO encapsulating VIP-SSL in **healthy** female Sprague-Dawley rat. Each value is Mean $\pm$ SEM, n = 3.

TISSUE	% I.D. per gram of tissue	
	SSL	VIP-SSL
Normal Breast	0.04 $\pm$ 0.00	0.04 $\pm$ 0.01
Heart	0.13 $\pm$ 0.04	0.18 $\pm$ 0.03
Lungs	0.20 $\pm$ 0.03	0.31 $\pm$ 0.02
Spleen	9.47 $\pm$ 0.96	9.84 $\pm$ 0.72
Liver	0.87 $\pm$ 0.14	0.88 $\pm$ 0.03
Blood	1.10 $\pm$ 0.23	0.96 $\pm$ 0.06
Kidneys	1.11 $\pm$ 0.13	2.42 $\pm$ 0.13
Muscle	0.02 $\pm$ 0.00	0.02 $\pm$ 0.01

Table 3: Biodistribution of Tc99m-HMPAO encapsulating SSL and Tc99m-HMPAO encapsulating VIP-SSL in **MNU-induced tumor** bearing rats. Each value is Mean $\pm$ SEM, n = 5.

TISSUE	% I.D. per gram of tissue	
	SSL	VIP-SSL
Breast tumor	0.13 $\pm$ 0.06	0.23 $\pm$ 0.02
Heart	0.09 $\pm$ 0.02	0.07 $\pm$ 0.02
Lungs	0.49 $\pm$ 0.32	0.28 $\pm$ 0.05
Spleen	10.36 $\pm$ 1.60	13.37 $\pm$ 1.19
Liver	0.83 $\pm$ 0.16	0.90 $\pm$ 0.19
Blood	1.44 $\pm$ 0.11	1.17 $\pm$ 0.26
Kidneys	1.02 $\pm$ 0.11	1.57 $\pm$ 0.24
Muscle	0.03 $\pm$ 0.01	0.01 $\pm$ 0.01

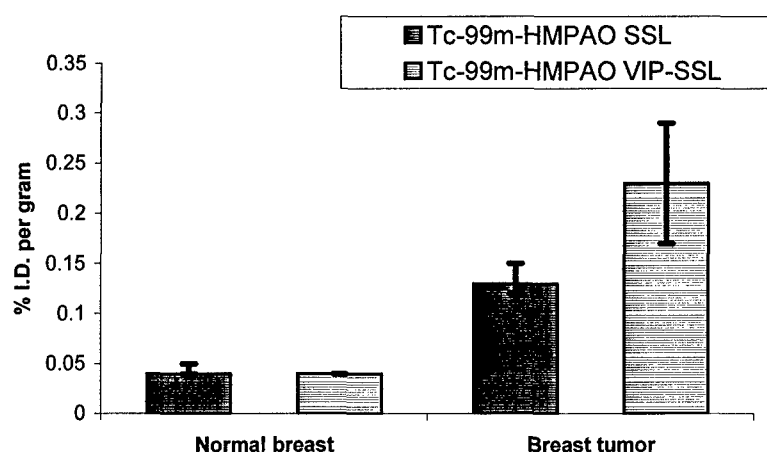


Figure 5. Accumulation of Tc-HMPAO loaded-VIP-SSL and SSL in normal breast tissue and breast cancer tissue. (Values are mean $\pm$ SEM, n=5). *p<0.05 in comparison to SSL in breast cancer; # p<0.05 in comparison to SSL and VIP-SSL in normal breast, ^ p<0.05 in comparison to SSL and VIP-SSL in normal breast

Status of Task 3: COMPLETED (Year 2)

Task 4: Determine the Pharmacokinetics of labeled VIP-SSL in Breast Cancer Bearing Rats (Year 2)

In order to demonstrate the steric stability of the developed liposomes pharmacokinetic studies were performed. Rats with induced mammary tumor were divided into two groups of five rats. Each group was injected with 300 μ Ci as Tc-labeled VIP-SSL, or SSL, by the tail vein. Two control rat groups without tumors (healthy rats) received SSL or Classical liposomes (non-PEG) similarly. Blood samples, 100 μ l each were withdrawn from a chronic carotid arterial cannula at 5 min, and 0.5, 1, 2, 4, 8, 12, and 24hr post administration. The radioactivity of each sample was measured and the blood profile of the liposomes determined. Pharmacokinetic parameters were then estimated using Win-Nonlin, version 1.5.

Results: The blood profile of Tc-99m-HMPAO encapsulating SSL in normal rats was evaluated and compared to the behavior of Tc-99m-HMPAO encapsulating liposomes without PEG, i.e. classical liposomes so as to determine whether these SSL are indeed sterically stabilized *in vivo* (Figure 6). Classical liposomes were cleared much faster than SSL and indicating that the presence of PEG on the liposomes did indeed increase the circulation half-life of the liposomes.

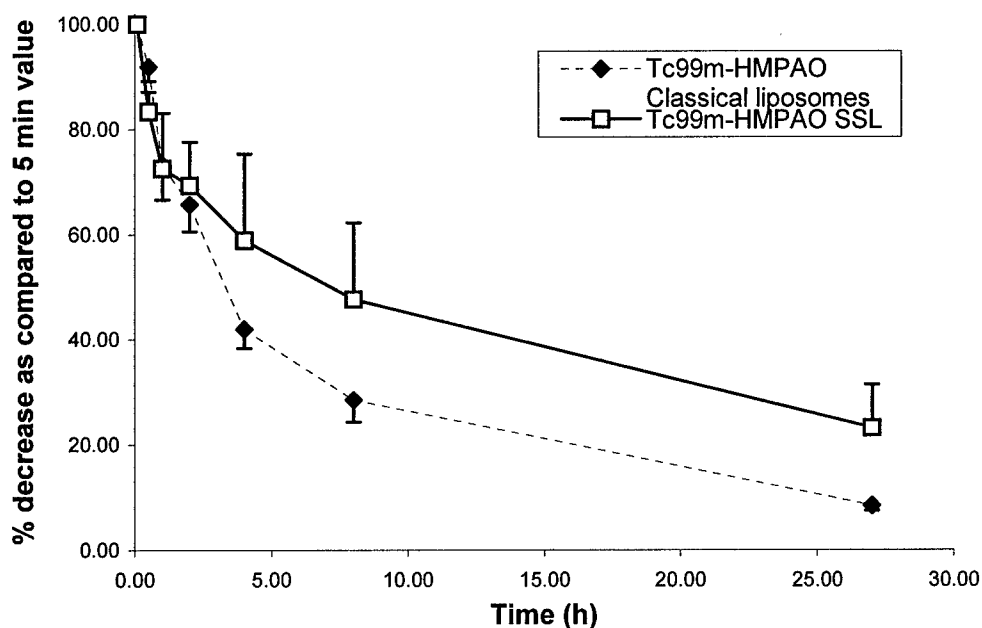


Figure 6: Blood profile of Tc-99m-HMPAO SSL and Tc-99m-HMPAO classical liposomes in normal healthy rats. Each value is mean $\pm$ SD, n = at least 3.

In tumor bearing rats, the decline in the amount of radioactivity in the blood with time was similar for both Tc-99m-HMPAO encapsulating SSL and Tc-99m-HMPAO

encapsulating VIP-SSL, over a 27 h period, as seen in Figure 7, suggesting that the presence of VIP on the surface did not alter the blood behavior of SSL.

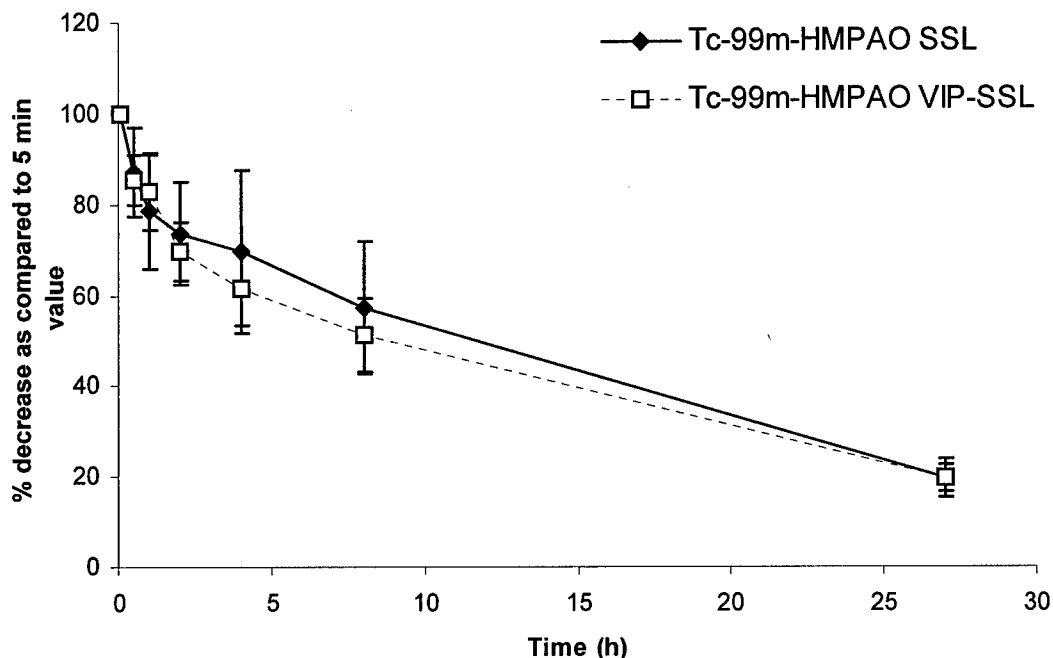


Figure 7: Blood profile of Tc-99m-HMPAO SSL and Tc-99m-HMPAO VIP-SSL in MNU-induced tumor bearing rats. Each value is mean $\pm$ SD, n = at least 5.

The pharmacokinetic parameters estimated from the blood profile data using Win-Nonlin, version 1.5 is shown in table 4. The half-life of about 14 -16 h for both Tc-99m-HMPAO encapsulating SSL and Tc-99m-HMPAO encapsulating VIP-SSL indicates the long-circulating nature of these liposomes. Moreover, various pharmacokinetic parameters calculated, were similar for both Tc-99m-HMPAO encapsulating SSL and Tc-99m-HMPAO encapsulating VIP-SSL, once again suggesting that the presence of VIP on the surface did not alter the pharmacokinetic characteristics of SSL.

This data clearly demonstrated that presence of VIP did not have any detrimental effect on the PK of SSL. This is most likely due to VIP being an endogenous peptide and the favorable interaction of VIP with lipids prevented removal from circulation by RES. The demonstration of long half-life of SSL-VIP is very important to ensure passive targeting with subsequent active targeting to breast tumors.

Status of Task 4: COMPLETED (Year 2)

Table 4: Pharmacokinetic Parameters of Tc99m-HMPAO SSL and Tc99m-HMPAO Classical Liposomes (CL) in normal rats and Tc99m-HMPAO VIP-SSL in tumor bearing rats calculated using Win-Nonlin version 1.5. Values are expressed as mean $\pm$ SD, n = 3-5.

Pharmacokinetic Parameters	Formulation			
	Normal Rat		Tumor Bearing Rat	
	Tc99m-HMPAO CL	Tc99m-HMPAO SSL	Tc99m-HMPAO SSL	Tc99m-HMPAO VIP-SSL
K_{el} (h ⁻¹)	0.09 $\pm$ 0.01	0.05 $\pm$ 0.01	0.05 $\pm$ 0.01	0.05 $\pm$ 0.01
$T_{1/2\beta}$ (h)	7.96 $\pm$ 0.97	15.87 $\pm$ 3.83	15.76 $\pm$ 3.90	13.89 $\pm$ 1.61
CL (L/h)	1.41 $\pm$ 0.18	1.02 $\pm$ 0.73	0.72 $\pm$ 0.16	0.77 $\pm$ 0.16

KEY RESEARCH ACCOMPLISHMENTS

1. The Tc-99m-HMPAO labeled sterically stabilized liposomes were successfully prepared with a mean diameter of about 110 nm and a high Tc-99m labeling efficiency of about 85%. (Year 1)
2. Vasoactive intestinal peptide (VIP) was successfully conjugated to DSPE-PEG₃₄₀₀ at the N-terminal amine of the peptide. This DSPE-PEG₃₄₀₀ conjugated VIP retained the bioactivity of the native VIP. (Year 1)
3. The DSPE-PEG₃₄₀₀ conjugated VIP was used to form Tc-99m-HMPAO labeled sterically stabilized liposomes surface modified with VIP (VIP-SSL). No significant leakage of the encapsulated Tc-99m-HMPAO label and no significant change in size, phospholipid content and labeling efficiency were observed due to the insertion process. This method was simple and ensured that all the VIP molecules are on the outer surface of the liposomes available for interaction with the receptors. (Year 1)
4. The *in vitro* targeting studies using breast cancer tissues and VIP-SSL with a non-exchangeable label, fluorescent cholesterol (BODIPY-Chol) incorporated in the bilayer, confirmed the successful binding of VIP-SSL to rat breast cancer *in vitro*. (Year 1)
5. Results of the biodistribution experiments demonstrated that there was significantly more accumulation of Tc-99m-HMPAO encapsulated SSL and Tc-99m-HMPAO

encapsulated VIP-SSL in breast tumor as compared to normal breast tissue demonstrating passive targeting. (Year 2)

6. A significant increase in the accumulation of Tc-99m-HMPAO encapsulated SSL-VIP in comparison to Tc-99m-HMPAO encapsulated SSL in rat breast tumor demonstrated success in active targeting to breast tumors. (Year 2)
7. The prepared SSL had significantly slower clearance rate than classical liposomes resulting in increased circulation half-life. (Year 2)
8. The presence of VIP did not make a significant difference in the pharmacokinetic behavior of the SSL. Both SSL and VIP-SSL had similar blood profile and pharmacokinetic parameters demonstrating longevity in circulation. (Year 2)

REPORTABLE OUTCOMES

Proceedings

Önyüksel, H., Dagar, S., Krishnadas, A., Blend, M., Rubinstein, I., "VIP-Liposomes for active, cell-specific targeted delivery to breast cancer in vivo", *NCI & CRS Second International Symposium on Tumor Targeted Delivery Systems*, Rockville, MD, 2002.

Manuscripts

Sumeet Dagar, Israel Rubinstein, Hayat Onyüksel, (2003), Liposomes in Ultrasound and Gamma Scintigraphic Imaging, *Methods in Enzymology*, 373:198-214

Sumeet Dagar, Aparna Krishnadas, Israel Rubinstein, Michael J Blend, Hayat Onyüksel, (2003), VIP grafted sterically stabilized liposomes for targeted imaging of breast cancer: in vivo studies. *J Control Release*, 91(1-2):123-33.

Pending Grants (based on work supported in this grant)

National Institute of Health (R01), Hayat Onyüksel (PI), "Improved Breast Cancer Chemotherapy with Actively Targeted Phospholipid Nanocarriers".

CONCLUSIONS

We have successfully conjugated VIP to DSPE-PEG₃₄₀₀ and incorporated this conjugate into preformed sterically stabilized liposomes to form a VIP-SSL construct. We have also shown the feasibility of this novel construct to actively target to MNU-induced rat breast cancer in vitro. This targeted carrier system was demonstrated to possess long circulation time in vivo leading to successful passive targeting and active targeting by VIP-R interaction to breast tumors resulting in significantly enhanced accumulation in the tumors.

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3. Dagar, S., Sekosan, M., Rubinstein, I. and Önyüksel, H.: Detection of VIP receptors in MNU-induced breast cancer in rats: Implications for breast cancer targeting. *Breast Cancer Research & Treatment* 65:49-54, 2001.

APPENDIX

1. Sumeet Dagar, Aparna Krishnadas, Israel Rubinstein, Michael J Blend, Hayat Onyuksel, (2003), VIP grafted sterically stabilized liposomes for targeted imaging of breast cancer: in vivo studies. *J Control Release*, 91(1-2):123-33.



VIP grafted sterically stabilized liposomes for targeted imaging of breast cancer: in vivo studies

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Abstract

Targeted delivery of radionuclides and therapeutic agents to specific biomarkers of breast cancer has important implications for the diagnosis and therapy of breast cancer. Vasoactive intestinal peptide receptors (VIP-R) are approximately five times more expressed in human breast cancer, compared to normal breast tissue. We have used VIP, a 28 amino acid mammalian neuropeptide, as a breast cancer targeting moiety for targeted imaging of breast cancer. VIP was covalently attached to the surface of sterically stabilized liposomes (SSL) that encapsulated a radionuclide, Tc99m-HMPAO. Rats with *n*-methyl nitrosourea (MNU)-induced in situ breast cancers were used to test this targeted liposomal imaging agent. Specifically, the pharmacokinetics and biodistribution of Tc99m-HMPAO encapsulating SSL with and without VIP were determined together with their ability to image breast cancer. The presence of VIP did not alter the size and Tc99m-HMPAO encapsulation ability of SSL. It also did not alter the pharmacokinetic profile of SSL. Long-circulating liposomes with and without VIP on their surface accumulated at significantly higher quantities in breast cancer when compared to normal breast, indicating passive targeting of these constructs to cancer tissues. Importantly, in breast cancer, Tc99m-HMPAO encapsulating SSL with VIP showed significantly more accumulation than SSL without VIP. The tumor to non-tumor ratio was also significantly higher for Tc99m-HMPAO encapsulating VIP-SSL than Tc99m-HMPAO encapsulating SSL without VIP, suggesting active targeting of VIP-SSL to breast cancer. Collectively, these data showed that Tc99m-HMPAO encapsulating VIP-SSL can be successfully used for the targeted imaging of breast cancer.

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Keywords: Tc99m-HMPAO; VIP liposome pharmacokinetics; VIP liposome biodistribution; Tumor targeting; Breast cancer imaging

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1. Introduction

Targeted delivery of radionuclides and therapeutic agents to cancer has important implications for detection, diagnosis and therapy of cancer. Bio-

markers that differentiate cancerous tissue from normal tissues can be used as targets for this purpose. One of these attractive molecular targets is vasoactive intestinal peptide receptors (VIP-R), which are overexpressed, about five times, in human breast cancer compared to normal breast tissue [1,2]. In vitro studies using human breast cancer tissues and cells have shown the presence of high densities of VIP receptors, with high affinity and specificity for VIP. Even though breast cancers are frequently known to be polyclonal, studies have shown that VIP receptors exist homogeneously in surgically resected human breast tumors and biopsies, both primaries and metastases. Therefore, VIP-R can be exploited to actively target carriers to breast cancer for effective diagnosis and therapy of breast cancer. In addition to active targeting, particulate carriers such as liposomes with mean size of about 100 nm are passively targeted by predominantly accumulating at certain disease sites such as cancer or inflammation due to the presence of leaky vasculature and liposomal extravasation.

The animal model we used in this study is an orthotopic model where the breast cancer is developed using a carcinogen, *n*-methyl nitrosourea (MNU). This model is more representative of human breast cancer than a xenograft model since the cancer develops in the breast without introduction of cancer cells exogenously. Recently, we have shown that VIP-R are approximately five times more overexpressed in rat breast cancer induced with MNU, similar to that observed in human breast cancer [3,4]. Previously, we have also developed a sterically stabilized liposomal carrier system with covalently attached active VIP on its surface and loaded with Tc99m-HMPAO [5]. The developed liposomal imaging agent encapsulates multiple molecules of Tc99m for high imaging sensitivity and its surface is modified with VIP for increased breast cancer specificity. The rationale behind our approach is depicted in Fig. 1.

Our long-term goal is to develop a targeted liposomal imaging agent for early detection of breast cancer. This study investigates the targeting ability of VIP grafted sterically stabilized liposomes (SSL) that encapsulates a radionuclide (Tc99m-HMPAO), in an animal model with fully developed breast cancer. The specific aim of this study was to

determine the cancer tissue accumulation and cancer image enhancement ability of these liposomes in rats with MNU-induced breast cancer. The results are also compared to similar SSL without VIP, in order to evaluate the active targeting to breast cancer by this novel carrier system.

2. Materials and methods

2.1. Materials

L- α -Egg yolk phosphatidylcholine type V-E in chloroform-methanol (9:1, v/v), cholesterol, glutathione and HEPES buffer were obtained from Sigma (St. Louis, MO, USA), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol from Sygena (Switzerland) and DSPE-PEG₃₄₀₀-NHS {1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[poly(ethylene glycol)]-*N*-hydroxy succinamide, PEG mw 3400} and polyethylene glycol (mw 2000) conjugated distearoylphosphatidyl ethanolamine (DSPE-PEG₂₀₀₀) were obtained from Shearwater Polymers (Huntsville, AL, USA). Vasoactive Intestinal Peptide (human/rat) was synthesized, using solid-phase synthesis, by the Protein Research Laboratory at the Research Resources Center, University of Illinois at Chicago. ELISA Kit for VIP was from Peninsula Laboratories (San Carlos, CA, USA). Chloroform HPLC grade and methanol HPLC grade were obtained from Fisher Scientific (Pittsburgh, PA, USA).

Freshly eluted Tc99m was obtained from the generator in the Section of Nuclear Medicine, University of Illinois Hospital. Hexamethyl propylene amine Oxime or Ceretec® was purchased from Amersham Healthcare (Arlington Heights, IL, USA). Virgin female Sprague-Dawley rats (~140 g, age 36 days old) were purchased from Harlan (Indianapolis, IN, USA).

2.2. Methods

2.2.1. Preparation of Tc99m-HMPAO encapsulating VIP-SSL and SSL

Tc99m-HMPAO encapsulating VIP-SSL were prepared as described previously [5]. First, Tc99m-HMPAO SSL were prepared by hydration of dried lipid film followed by extrusion, as described before

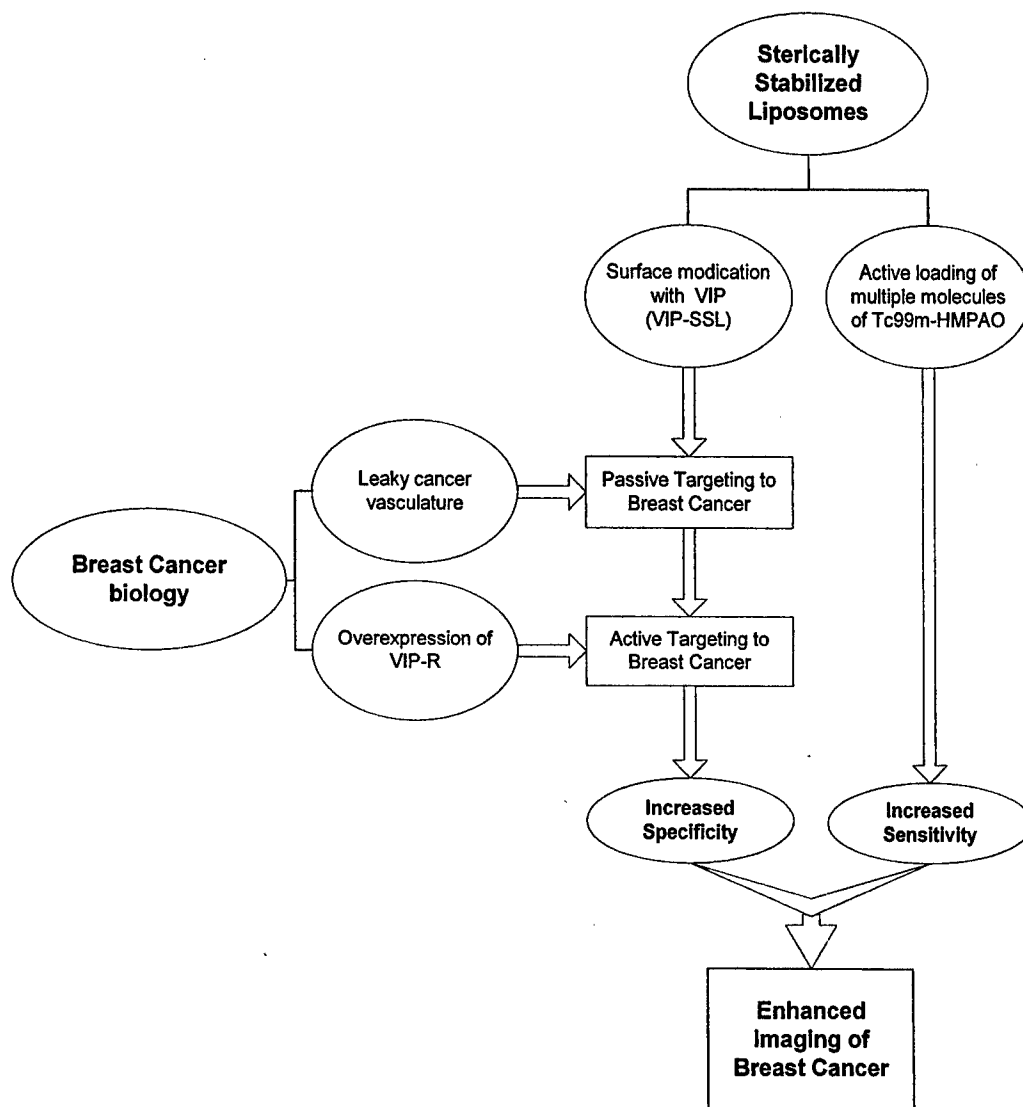


Fig. 1. Flow-chart depicting the novel approach for targeted breast cancer detection.

[6,7] with modifications. Briefly, egg-phosphatidylcholine (PC), cholesterol (CH), polyethylene glycol (molecular weight 2000) conjugated distearyl phosphatidylethanolamine (DSPE-PEG) and dipalmitoyl phosphatidylglycerol (DPPG) were dissolved in an organic solvent and solvent evaporated to form a dry film, which was hydrated with isotonic, 50 mM glutathione containing isotonic 0.01 M HEPES buffer (pH 7.4), and extruded through a polycarbonate filter to reduce the size. The untrapped glutathione

was removed by gel filtration and the liposomes immediately labeled with Tc-99m-HMPAO using an efficient Tc-99m loading procedure that we have adapted [8,9], with modifications [5,6]. DSPE-PEG₃₄₀₀-VIP was inserted into Tc99m-HMPAO SSL by incubation at 37 °C. The free DSPE-PEG₃₄₀₀-VIP and the label were then removed by gel filtration to give Tc99m-HMPAO VIP-SSL. The Tc99m-HMPAO encapsulating SSL were prepared in a similar manner, except 5 mol% of DSPE-PEG was

used instead of 3 mol% for Tc99m-HMPAO encapsulating VIP-SSL and the radiolabeled SSL were not incubated with DSPE-PEG₃₄₀₀-VIP after separation of free Tc99m-HMPAO.

2.2.2. Characterization of Tc99m-HMPAO encapsulating VIP-SSL and SSL

The sizes of both Tc99m-HMPAO SSL and Tc99m-HMPAO VIP-SSL were determined by quasi-elastic light scattering using a NICOMP Particle Sizer Model 370 (Particle Sizing Systems, Menlo Park, CA, USA). The radioactivity content and the labeling efficiency were determined by counting the radioactivity of each fraction collected, along with radioactivity remaining in the column, using a dose calibrator, and calculating the percentage of radiolabel present in the liposomes versus the total radioactivity. The phospholipid content of the Tc-99m-HMPAO encapsulating liposomes was determined by the modified Bartlett phosphate assay [10]. The VIP content of Tc99m-HMPAO encapsulating VIP-SSL was determined by a commercial ELISA kit.

2.2.3. In vivo studies

Animal studies were carried out in accordance with the Institutional Animal Care Committee guidelines and the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

Female rats with MNU-induced mammary cancer were used as the animal model for the in vivo studies. Breast cancer was induced in virgin female Sprague–Dawley rats with MNU as previously described in the literature with some modification [4,5,11,12]. Briefly, virgin female Sprague–Dawley rats, 36 days old, weighing ~140 g, were anesthetized with ketamine/xylazine (13.3/1.3 mg per 100 g body weight, i.m.). Each animal received a single intravenous injection of MNU (50 mg/kg body weight) in acidified saline (pH 5.0), via the tail vein. The rats were weighed weekly. They were palpated every week, starting at 3 weeks post-MNU administration. Palpable mammary cancer was detected within 100–150 days after injection. The cancer-bearing rats, with an average tumor size of

about 2 cm diameter, were divided into two groups of at least five rats each. Each group was assigned to receive one of the radiotracers: Tc99m-HMPAO VIP-SSL or Tc99m-HMPAO SSL. The same group of rats was used to perform the pharmacokinetic, biodistribution and imaging studies as detailed below.

2.2.4. Pharmacokinetic studies

These studies evaluated the behavior of labeled SSL with or without VIP on their surface in the blood. Rats were anesthetized with ketamine/xylazine (13.3/1.3 mg per 100 g body weight, i.p.). After anesthesia, the carotid artery was exposed and cannulated with a polyethylene cannula (PE-50) for blood sampling. The cannula was exteriorized near the back of the neck. It was then filled with Heparin solution to reduce the chance of blockage due to clotting. The incision used for exposure of the carotid artery was closed with wound clips. The cannulated rats were then given ~300 μ Ci of the assigned radiotracer (Tc99m-HMPAO encapsulating VIP-SSL or Tc99m-HMPAO encapsulating SSL) intravenously via the tail vein (at least five rats per group). 100 μ l blood samples were then withdrawn from the chronic carotid artery cannula at 5 min and then at 0.5, 1, 2, 4, 8 and 27 h after injection. The radioactivity of each blood sample was measured in a gamma counter (Cobra 5005, Packard Instruments). The change in the amount of radioactivity in the blood at each sampling time point (compared with the 5 min sample) after intravenous injection of Tc-99m-HMPAO encapsulating VIP-SSL or SSL was calculated.

2.2.5. Imaging studies

These experiments evaluated the breast cancer image enhancement ability of Tc-99m-HMPAO encapsulating VIP-SSL or SSL using cancer-bearing rats. Imaging studies were performed as described before with appropriate adjustments [9] and on the same rats as those used for pharmacokinetic studies.

Briefly, the rats were anesthetized with ketamine/xylazine and then the appropriate radioactive formulation (~300 μ Ci/rat) was administered intravenously by tail vein. The rats were imaged at ~3 and ~27 h post-injection. For imaging the rats were again anesthetized and placed prone on one head of a

triple-headed Picker PRISM 3000 SPECT gamma camera equipped with a low-energy, high-resolution collimator and a dedicated Odyssey computer. The images (100,000 counts/image) were acquired and stored in a 512×512 matrix.

The Odyssey software program was used to analyze relative uptake in cancer tissues and the background tissue (calf muscles) in the same animal. The images were analyzed by drawing regions of interest (ROIs) over the breast tumors and calf muscles as background. The uptake was measured as counts per pixel. The uptake in the two calf muscles and the tumors, if more than one tumor per rat, were averaged. Counts per pixel values for five animals were calculated as mean±S.E.M. Tumor tissue to background (or tumor to non-tumor, T-NT) ratios were calculated. Statistical analysis was performed using unpaired Student's *t*-test to compare different formulations. The level of significance was set at $P<0.05$.

2.2.6. Biodistribution studies

These studies investigated the tissue distribution of Tc99m-HMPAO encapsulating SSL or VIP-SSL after systemic delivery to rats and the effect of the presence of VIP on the surface of SSL on their biodistribution.

The MNU-induced rats used were the same as those used for the pharmacokinetic studies described above. About 300 μ Ci of Tc99m-HMPAO encapsulating VIP-SSL or Tc99m-HMPAO encapsulating SSL was injected into the tail vein of the rat. At 27 h post-injection, the rats were euthanized by an overdose of ketamine/xylazine. A blood sample was obtained by cardiac puncture. Tissues (normal breast or breast cancer, liver, spleen, kidneys, calf muscle, heart and lungs) were dissected out. They were

washed with saline, dried between folds of paper towel and transferred to pre-weighed polypropylene tubes and capped. The tubes were then weighed and the weight of each of the tissues was determined. Their activity was measured in a shielded well scintillation gamma counter (Cobra 5005, Packard Instruments). To correct for the physical decay of Tc99m, and to permit calculation of the uptake of the radiolabeled liposomes, a 10 μ l aliquot of the injected dose was also counted. The results were expressed as percent injected dose per gram of tissue (% I.D./g).

The tissue radioactivity amounts, % I.D./g, were compared between formulations (Tc-99m-HMPAO encapsulating SSL or Tc-99m-HMPAO encapsulating VIP-SSL) as well as between normal and tumor-bearing rats for each of the formulations using unpaired Student's *t*-test. $P<0.05$ was considered significant.

3. Results

3.1. Characteristics of Tc99m-HMPAO encapsulating VIP-SSL and Tc99m-HMPAO encapsulating SSL

Tc99m-HMPAO encapsulating VIP-SSL with a high Tc-99m-HMPAO labeling efficiency were prepared in a reproducible manner. The characteristics of Tc99m-HMPAO encapsulating VIP-SSL and Tc99m-HMPAO encapsulating SSL were not significantly different from each other as shown in Table 1. The size of both Tc99m-HMPAO encapsulating VIP-SSL and SSL was around 110 nm. The phospholipid content of both Tc99m-HMPAO encapsulating VIP-SSL and SSL was about 3.0 μ mol/

Table 1
Characteristics of Tc99m-HMPAO encapsulating VIP-SSL and SSL (each value is mean±standard deviation, n =at least 6)

Characteristic	Tc99m-HMPAO encapsulating SSL	Tc99m-HMPAO encapsulating VIP-SSL
Size (nm)	109.81±14.21	114.77±13.72
Phospholipid content (μ mol/ml)	3.15±0.23	3.01±0.48
Radioactivity content (μ Ci/ml)	1008±160	800±113
Labeling efficiency (%)	85.7±4.46	83.8±2.42
VIP content (μ g/ μ mol phospholipid)	N/A	10.5

ml. The radioactivity content of both Tc99m-HMPAO encapsulating VIP-SSL and SSL was about 900 $\mu\text{Ci/ml}$. In addition, both the liposomes showed a high labeling efficiency of about 85%.

The VIP content of Tc-99m-HMPAO encapsulating VIP-SSL was found to be 10.5 $\mu\text{g}/\mu\text{mol}$ phospholipid, which translates to approximately 225 VIP molecules per liposome.

3.2. Pharmacokinetic studies

The percent decrease in radioactivity with time was used to monitor the circulation time of liposomes. The radioactivity at 5 min was considered to be 100%. The decline of radioactivity in the blood with time in breast tumor-bearing rats was similar for both Tc99m-HMPAO encapsulating SSL with and without VIP, over a 27 h period, as seen in Fig. 2. The figure also shows that the decline of radioactivity for both formulations was largely monophasic. In addition, the pharmacokinetic parameters calculated from the blood radioactivity data using Win-Nonlin (data not shown) indicated that the half-lives (~ 16 h) of Tc99m-HMPAO encapsulating VIP-SSL and

Tc99m-HMPAO encapsulating SSL were not significantly different from each other.

3.3. Imaging studies

The images acquired for both Tc-99m-HMPAO encapsulating VIP-SSL and Tc-99m-HMPAO encapsulating SSL injected into cancer-bearing rats at about 3 h and 27 h post-injection were analyzed using the counts per pixel on the stored images using commercial Odyssey software. As seen in Fig. 3, in all cases, the T-NT ratio was over 4 and for both SSL with and without VIP image enhancement (T-NT ratio) increased at the later imaging time. For both imaging times (3 and 27 h) SSL with VIP showed significantly higher image enhancement (higher T-NT ratio) than liposomes without VIP, as determined by unpaired Student's *t*-test.

3.4. Biodistribution studies

This study was conducted to determine the uptake of SSL and VIP-SSL by various tissues in tumor-bearing rats. In particular, breast cancer tissue was compared with normal breast tissue.

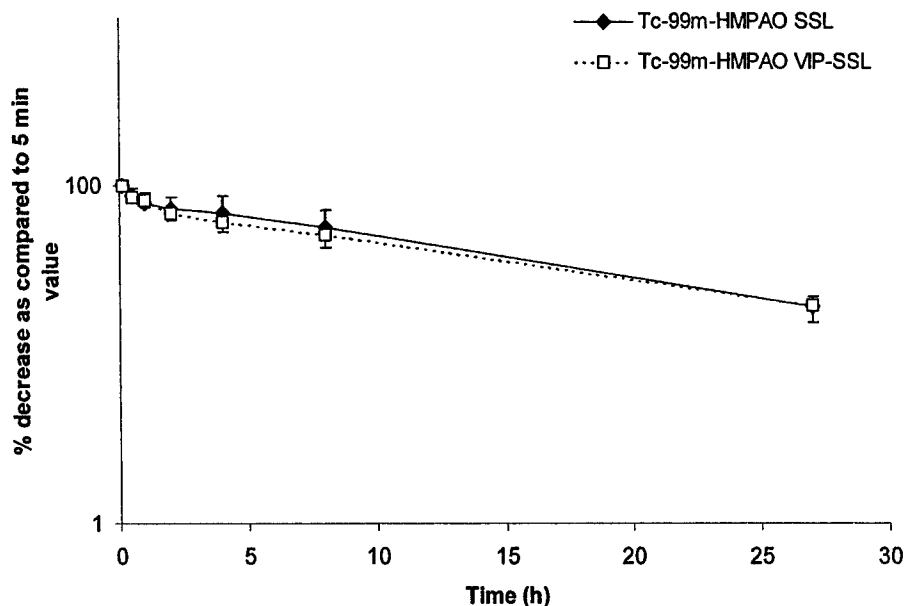


Fig. 2. Change in radioactivity in blood with time of Tc-99m-HMPAO VIP-SSL and Tc-99m-HMPAO SSL after injection in MNU-induced cancer-bearing rats. Each value is mean $\pm$ S.D., n =at least 5.

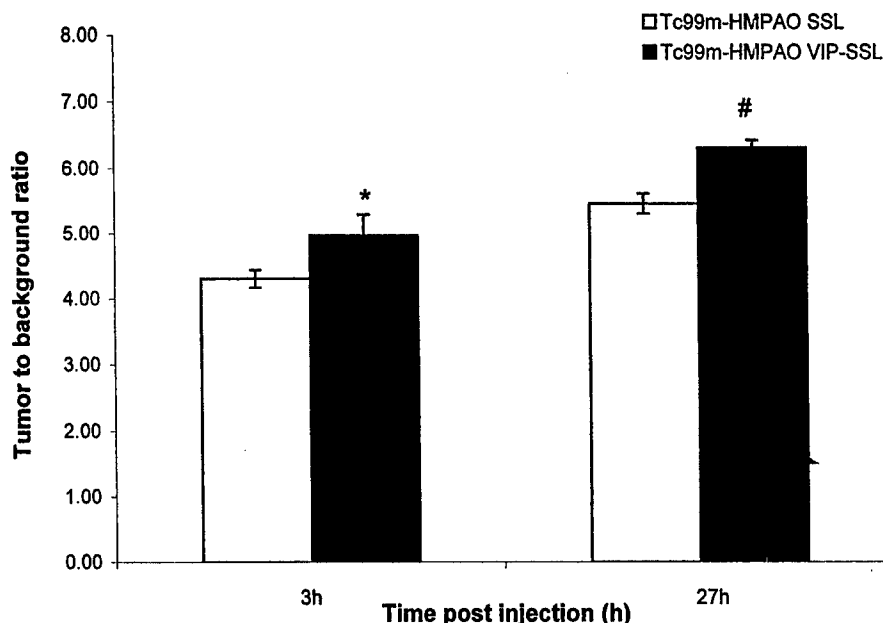


Fig. 3. Tumor to background ratio of Tc-99m-HMPAO encapsulating SSL with or without VIP at ~3 and ~27 h post-injection. Data are expressed as mean $\pm$ S.E.M., n =at least 5. * P <0.05 as compared to Tc99m-HMPAO encapsulating SSL at ~3 h post-injection; # P <0.05 as compared to Tc99m-HMPAO encapsulating SSL at ~27 h post-injection.

The mean breast cancer uptake of Tc99m-HMPAO encapsulating VIP-SSL was about 1.8 times more compared to the cancer uptake of Tc99m-HMPAO encapsulating SSL (Fig. 4). In addition, there was significantly more uptake of VIP-SSL in kidneys as compared to SSL in tumor-bearing rats (Table 2). There was no significant difference in the accumulation of Tc99m-HMPAO encapsulating SSL and Tc99m-HMPAO encapsulating VIP-SSL in the other tissues analyzed (Table 2).

4. Discussion

The presence of DSPE-PEG on the surface and the mean diameter of ~110 nm ensured the long-circulating (half-life ~16 h) capability of our liposomal constructs and maximized their potential for passive targeting to breast tumors, most probably due to the "Enhanced Permeability and Retention" or EPR effect [13–15]. The loading efficiency of these liposomes was high (~85%). The label used, Tc99m, is the most preferable label for diagnostic applications [16,17] because of its relatively short

half-life of 6.02 h, monoenergetic emission of 140 keV, lack of alpha or beta emission, low cost and widespread availability. Furthermore, the uniqueness of this system is that it is able to deliver multiple numbers of radionuclides to each target (VIP-R), hence increasing the sensitivity of detection. The covalent attachment of a peptide on the liposome surface without significant alteration of the labeled liposomes may indicate that similar liposomes carrying chemotherapeutic agents can be prepared to target the VIP receptors of breast or other cancers that overexpress VIP-R for targeted chemotherapy.

Both Tc99m-HMPAO encapsulating SSL and Tc99m-HMPAO encapsulating VIP-SSL showed a monophasic decline, characteristic of long-circulating liposomes, with no significant difference in half-lives (half-lives ~16 h). Similar to the *in vitro* characteristics, these data suggest that the presence of VIP on the SSL does not alter the long-circulating behavior of the liposomes. This finding is in good agreement with the literature. It has been shown that non-antibody ligands such as peptides when coupled to SSL do not significantly alter the circulation times of ligand-free SSL [18,19]. The long circulation

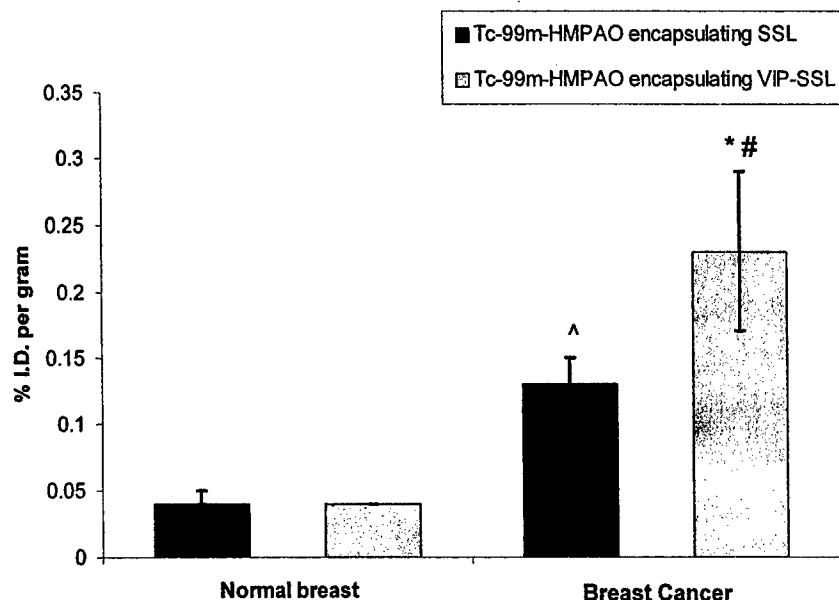


Fig. 4. Accumulation of Tc-99m-HMPAO encapsulating VIP-SSL and Tc-99m-HMPAO encapsulating SSL in normal breast tissue and breast cancer tissue (each data, $n=5$, mean $\pm$ S.E.M.). * $P<0.05$ compared to Tc-99m-HMPAO encapsulating SSL in breast cancer; # $P<0.05$ compared to Tc-99m-HMPAO encapsulating SSL and VIP-SSL in normal breast; $\times P<0.05$ compared to Tc-99m-HMPAO encapsulating SSL and VIP-SSL in normal breast.

half-life of Tc-99m-labeled VIP-SSL along with their small size (~ 114 nm) make these liposomes suitable for accumulation in breast cancer due to the EPR effect.

The tumor to background (T-NT) ratio was better at 27 h post-injection than at 3 h post-injection for both formulations (Fig. 3), which can be explained by the EPR effect. It is known that, for particulate

systems, more accumulation occurs into the areas of leaky vasculature with time and a more particulate imaging agent will sequester at the cancer site more than the background tissues, improving the tumor to non-tumor ratio and facilitating the detection of the cancer [14]. This increased T-NT ratio is indicative of long circulation in the blood and passive targeting of SSL to breast cancer. In addition, the tumor to non-tumor (T-NT) ratios for Tc99m-HMPAO encapsulating VIP-SSL were significantly ($P<0.05$) more than that for Tc99m-HMPAO encapsulating SSL, suggesting active targeting of VIP-SSL, most probably due to the interaction of liposomal VIP and VIP-R in the breast cancer. The T-NT ratio for Tc99m-HMPAO encapsulating VIP-SSL about 27 h post-injection was about 6:1, which is a clinically acceptable T-NT ratio for the tumor to be detected by planar gamma scintigraphy. This suggests that the targeted liposomes developed in this study can be successfully used to detect breast cancer in the clinic.

In agreement with imaging data, there was significantly more accumulation of both Tc99m-HMPAO encapsulating SSL and VIP-SSL in breast cancer as compared to normal breast tissue in rats,

Table 2
Biodistribution of Tc99m-HMPAO encapsulating VIP-SSL and Tc99m-HMPAO encapsulating SSL in MNU-induced cancer-bearing rats. Each value is the mean $\pm$ S.E.M., $n=5$

Tissue	% I.D. per gram of tissue	
	SSL	VIP-SSL
Heart	0.09 $\pm$ 0.02	0.07 $\pm$ 0.02
Lungs	0.49 $\pm$ 0.32	0.28 $\pm$ 0.05
Spleen	10.36 $\pm$ 1.60	13.37 $\pm$ 1.19
Liver	0.83 $\pm$ 0.16	0.90 $\pm$ 0.19
Blood	1.44 $\pm$ 0.11	1.17 $\pm$ 0.26
Kidneys	1.02 $\pm$ 0.11	1.57 $\pm$ 0.24*
Muscle	0.03 $\pm$ 0.01	0.01 $\pm$ 0.01
Breast cancer	0.13 $\pm$ 0.06	0.23 $\pm$ 0.02 [#]

* $P<0.05$ compared with Tc99m-HMPAO encapsulating SSL;

[#] $P<0.05$ compared with Tc99m-HMPAO encapsulating SSL.

suggesting that the liposomes were passively targeted to the breast cancer, most probably due the presence of leaky vasculature in the tumor (Fig. 4). Similarly, in breast cancer, significantly more accumulation of Tc99m-HMPAO encapsulating VIP-SSL as compared to Tc99m-HMPAO encapsulating SSL confirms that the liposomes were indeed actively targeted to the breast tumor (Fig. 4). The variability in the data obtained for the accumulation of labeled VIP-SSL was more than for labeled SSL without VIP in breast cancer. Since VIP-SSL are both passively and actively targeted to breast cancer, the variability in actively targeted Tc99m-HMPAO encapsulating VIP-SSL may be related to the developmental stage of the cancer and the extent of VIP-R expression at that stage. There is some evidence in the literature that suggests the differential expression of genes that code for proteins during MNU-induced breast cancer growth [20]. The same differential expression may also be true for VIP-R in breast cancer. Hence, active targeting to VIP-R showed increased variability depending on the VIP-R expression level of the breast cancer stage at which the particular cancer-bearing rat was used for this study.

There was no significant difference in the accumulation of labeled SSL with and without VIP in all other tissues (heart, liver, spleen, blood and muscle), except for the kidneys. The higher uptake by the kidneys may be related to the reduced flexibility of the VIP-SSL membrane, due to the insertion of VIP in the SSL bilayer. This most likely causes the entrapment of these liposomes in the vascular bed of the kidneys. However, more studies are needed to elucidate this matter.

The spleen showed the most prominent uptake ($\sim 10\%$ I.D./g) of all the tissues analyzed in both normal and tumor-bearing rats. These values fall in the range (8.53 to 16.25% I.D./g) reported in the literature for spleen uptake of Tc99m-HMPAO encapsulating SSL of about 100 nm in diameter [9,21,22]. In addition, this uptake was much less than the splenic uptake ($\sim 50\%$ I.D./g) observed for Tc99m-HMPAO encapsulating classical liposomes of similar size [23], indicating the steric stabilization of these liposomes.

In this study the percent accumulation of injected dose of liposomes in breast cancer tissue ($\sim 0.2\%$)

was several orders of magnitude less than those reported in the literature (2 to 20% per gram of tissue) [15,24–27]. The reasons for this discrepancy are unclear, but the data reported in the literature were obtained using xenograft tumors in nude mice, while values for this study were obtained from a MNU-induced *in situ* breast cancer rat model. Xenograft models do not represent the right environment that occurs in human tumors because, in xenograft models, the cells are already transformed when they are implanted in the mice, leading to shorter doubling times than observed in human cancer. Therefore, it is possible that less necrosis and a more aggressive blood supply exist within xenograft models [28]. This would lead to more extensive accumulation in the xenograft model when compared to human cancer [29]. MNU-induced breast cancer is a more representative model of human breast cancer, developing in the breast and not at the injection site and developing at a more realistic, slow rate. Therefore, due to the slow growth and the possibly larger areas of necrosis, the accumulation of liposomes in the cancer may be less with MNU-induced animals.

Active targeting to breast cancer can be further improved by taking into consideration the complex interplay of tumor biology (such as maximum VIP-R overexpression), product (such as optimum concentration of grafted VIP on SSL) and imaging procedure-related factors (such as optimum tumor imaging time post-injection).

5. Conclusion

Labeled, long-circulating, small liposomes with and without VIP accumulated significantly more in the tumor as compared to normal breast tissue, indicating passive targeting of SSL to breast cancer. There was significantly more accumulation of Tc99m-HMPAO labeled SSL surface grafted with VIP as compared to similar liposomes without VIP in breast tumors, indicating the successful use of VIP-R as molecular targets and the occurrence of active targeting of these liposomes to breast cancer in addition to passive targeting. Tumor accumulation of both the liposomes increased with time. Even though the image enhancement (T-NT ratio) observed in this study is considered sufficient for

tumors to be detected by planar scintigraphy in a clinical setting, the described novel targeted imaging technique can be further improved when images are taken at a tumor development stage with maximum VIP-R overexpression and tumor leakiness and by optimizing the product and imaging conditions. In addition, this targeted carrier system can be used for the imaging of other tumors that overexpress VIP-R such as prostate and endometrial cancers. This targeted carrier system is currently being evaluated in our laboratory for further improvement in imaging and also for targeted therapy of breast cancer with the use of VIP-SSL that encapsulate chemotherapeutic agents.

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