Specific Aims

Melanoma is the major cause of skin cancer mortality in the world. In contrast to many other malignancies the incidence and mortality trends have continued to increase over the last decade [2], despite the identification of numerous tumor-related biomarkers [3-6]. Melanoma initially develops in a radial fashion, followed by vertical growth, then invasion and metastasis. At each phase the prognosis and prediction of response to therapy cannot be determined fully based on primary tumor clinical parameters such as tumor site, thickness, ulceration, or mitotic rate. Therefore, additional data such as analysis of sentinel lymph node biopsies are required. Identification and measurement of novel tumor-related and serum biomarkers can provide additional information that may enhance the diagnosis and prognostication. The key to survival in patients with melanoma is early and accurate diagnosis and staging so that appropriate treatment regimes can be initiated.

Heparan sulfate proteoglycans are signal transducing and architectural proteins with diverse sulfated oligosaccharide chains found ubiquitously on all cells and extracellular matrices. HSPG molecules such as CD44, syndecan-1, glypican-1 and perlecan, that in tumors may be over-expressed or possess unique sulfation patterns, play a pivotal role in cell-cell adhesion, angiogenesis, and signaling and are therefore critical for the progression and metastasis of malignant cells [14]. Indeed, down regulation of glypican-1 [13] or perlecan [37] leads to decreased metastatic activity and invasion of melanoma cells, respectively. We have identified a class of peptides that bind to hypersulfated glycosaminoglycans (GAGs). These peptides when radioiodinated and injected *iv*, bind to and localize in B16F10 melanoma tumors grown as pulmonary metastases in the lung of syngeneic mice. They also identified tumor cell masses in the thoracic cavity, within adjacent lymph nodes and at subcutaneous sites. In contrast, these peptides do not bind significantly to healthy cells or tissues. Stemming from our previous work with HSPGs associated with pathologic amyloid deposits, it is hypothesized that peptide binding to tumors is mediated by uncommon hypersulfated HSPGs expressed by the melanoma cells. Furthermore the peptides may be used as reagents: **I)** to target tumor cells specifically *in vivo*, and **II)** for histochemical staining of tumor cells within formalin-fixed tissue sections.

In addition to validating hypersulfated HSPG as a novel biomarker for melanoma our studies will result in the identification and production of specific peptides optimized for *in vivo* diagnostic imaging and *ex vivo* tissue analysis for prognostication and ultimately for monitoring response to therapy.

Our aims are thus to:

I) Determine the optimal peptide from a panel of ~ 10 for melanoma tumor targeting *in vivo* using the murine model of B16F10 pulmonary metastases. To achieve this we will:

a) Examine and quantitatively compare the uptake of ^{125}I-labeled peptides in B16F10 lung colonies and lymph node metastases by using small animal SPECT/CT imaging, tissue biodistribution measurements and quantitative micro-autoradiography.

b) Compare, by using small animal PET/CT the binding of the 3 most efficacious ^{125}I-labeled peptides with that of the tumor imaging agents 2-deoxy-2-(^{18}F)fluoro-D-glucose (FDG) and 3'-deoxy-3'-(^{18}F)fluorothymidine (FLT) in the B16F10 lung model.

II) Examine, by histochemical staining, the reactivity of biotinylated peptide with formalin-fixed sections of canine and human primary and metastatic melanoma tissues as well as well as control non-diseased tissue sections.

III) Compare in the same animal, the tumor imaging of the most efficacious peptide (^{99m}Tc-labeled) with the PET/CT images of FDG using a surrogate model of human melanoma, i.e., dogs with naturally occurring metastatic melanoma.

Research Strategy

(a) **Significance**

1. Melanoma

Melanoma is the major cause of skin cancer mortality in the world. In contrast to many other malignancies the incidence and mortality trends have continued to increase over the last decade [1, 2], despite the identification of numerous tumor-related biomarkers [3-6]. In basic terms, melanoma develops in 3 phases – radial then vertical growth followed by metastasis. At each phase the prognosis and prediction of response to therapy cannot be determined fully based on clinical parameters such as tumor site, appearance, thickness, ulceration, or mitotic rate. Therefore, additional factors such as analysis of sentinel lymph node biopsies and the identification and measurement of novel tumor-related and serum biomarkers are sought. These additional data can provide a more detailed and exact clinical picture, which is imperative because the key to survival in patients with melanocyte-derived malignancies is early and accurate diagnosis and staging of the lesion [7, 8].

2. Role of heparan sulfate proteoglycans in (cancer and) melanoma

Heparan sulfate proteoglycans (HSPGs) are sulfated glycoproteins that contain unbranched covalently attached glycosaminoglycans (GAG) comprising modified N-acetylglucoasmine and glucuronic acid and are found in the extra-cellular matrix or cell surface of all cells and tissues [9, 10]. HSPGs, due to the inherent structural variability of their GAG chains, bind many ligands and thereby mediate signal transduction, e.g., fibroblast growth factor, epidermal growth factor, Hedgehog and many other angiogenic factors. The extra cellular matrix HSPG, perlecan, is associated with the progression and persistence of pathologic amyloid deposits, such as those formed from Aβ in the brains of patients with Alzheimer's disease. HSPGs are also over-expressed in many cancers including melanoma [11], where they have been correlated with cancer progression and poor prognosis. O'Connell *et al*. demonstrated a positive correlation between HSPG expression and the metastatic potential of UACC647 melanoma cells and linked this correlation to Wnt5A signaling potentiation [11]. Chondroitin sulfate (CS)-reactive antibodies have been used to demonstrate altered serological properties of the CS proteins in the extra-cellular matrix that discerned metastatic melanoma from atypical nevi [12], In addition, the HSPGs, CD44, perlecan and glypican1 have all been shown to mediate angiogenesis or enhance metastatic potential of melanoma cells *in vivo* and *in vitro* [13-15]. The varied biochemical nature of the HSPGs (chiefly, the extent and pattern of sulfation) that allow them to mediate different biological functions also make them difficult to measure and evaluate. We have shown, through our amyloid research that certain basic peptides are capable of selectively binding to amyloid-associated HSPG due to the fact that they are hypersulfated [16]. This same class of peptides now has been found to bind B16F10 melanoma lung colonies in mice as evidenced by small animal imaging and microautoradiography (see Fig. 2 Approach Section). This advance provides a non-invasive method to quantify the HSPG biomarker in melanoma and determine the correlation between biomarker expression and prognosis of primary and metastatic tumors. We have hypothesized therefore, that the expression of the novel melanoma biomarker identified by our peptides, and based on the accepted importance of HSPG in this disease, may have prognostic and diagnostic relevance for patients with metastatic melanoma.

3. Importance of Anatomic and Molecular Imaging

Both functional and anatomic imaging modalities are used to evaluate and stage patients with melanoma. Since the prognosis for patients with melanoma is based principally upon depth of invasion, ulceration and mitotic rate of the primary lesion, sentinel lymph node status, as well as the presence and site (local or distant) of metastases the use of imaging techniques provides important clinical information. The presence of metastasis to the sentinel lymph nodes is an important negative prognostic marker and it is therefore imperative to assess nodal involvement in patients with AJCC clinical stage I/II disease [17]. The most commonly used whole body imaging modality for detecting nodal, bone, and soft tissue anatomic anomalies is x-ray computed tomography (CT), which routinely

focuses on chest and abdomen imaging of patients. Mohr *et al*. recommend CT be limited however to patients with melanoma of AJCC stages IIC, IIIB, IIIC, and IIIA (with a macroscopic serial lymph node) [17]. In cases of suspected brain or neurological metastases magnetic resonance imaging (MRI) was recommended due to the higher tissue contrast and spatial resolution that afford advantages over CT imaging [18].

The use of x-ray, ultrasound, CT and MRI provide valuable anatomic data regarding the structure of the tumor and metastases; however, they provide no data on the metabolic state nor the molecular phenotype (expression of cell surface or cytosolic molecules of interest) of the lesion. The advent of metabolic imaging by positron emission tomography in conjunction with CT (PET/CT) using 2-deoxy-2-(^{18}F)fluoro-D-glucose (FDG) has drastically altered how malignancies are detected and monitored. PET/CT is not generally recommended for the detection of lesions < 1.0 cm but it has demonstrated greater specificity and sensitivity for the detection of metastatic melanoma than CT or PET alone [19, 20]. Furthermore, the metabolic status of the tumors can be monitored based upon the FDG uptake which is a function of both cell surface expression of the glucose transporter protein (GLUT1) and cytoplasmic hexokinase activity [21]. FDG is routinely used for imaging the hypermetabolic state of malignancies; however there are certain limitations to this technique that have encouraged the development of novel melanoma-specific imaging agents, based on targeting specific biomarkers present on the surface of the tumor [22].

Of the experimental radiotracers for imaging metastatic melanoma, α-melanocyte stimulating hormone (αMSH) is one of the most commonly employed [23, 24]. This 13 amino acid melanotropic peptide binds specifically to the melanocortin MC1 receptors that are over-expressed on the surface of human and murine melanoma cells. This peptide has been successfully labeled with technetium-99m (^{99m}Tc), yttrium-86 (^{86}Y), indium-111 (^{111}In), copper-64 (^{64}Cu), gallium-67 (^{67}Ga), and fluorine-18 (^{18}F) and used for both imaging and radiotherapy of melanoma tumors *in vivo* [25-29]. The radio-imaging with αMSH benefits from being melanoma specific and therefore potentially provides increased specificity and sensitivity as compared to FDG imaging.

In addition to the αMSH peptide, numerous other small molecules are being developed as imaging agents for malignant melanoma metastases that target either neo-expression of proteins (biomarkers) often on the cell surface or increased concentrations of normally expressed molecules; however, none of these reagents target the over-expression of native (or modified) HSPG in the tumor environs, as our peptides do. The identification of novel biomarkers associated with the progression and metastatic potential of melanoma will permit the development of targeting agents that can be used to image the distribution of lesions within the patient. In addition, each targeting agent may provide information on the molecular phenotype and metabolic state of the primary tumor or metastases, e.g. presently imaging can potentially inform the clinician about the expression of GLUT1 and hexokinase activity (FDG), MC1 receptor density (αMSH), and DNA replication (FLT) within each tumor. Thus the identification of novel, tumor-related biomarkers and identification of their expression during the course of disease progression can yield functional information and potential therapeutic approaches that may be disease or stage specific.

(b) **Innovation**

Sulfated proteoglycans are recognized as important mediators of pro-angiogenic and pro-metastatic signaling and are over-expressed by numerous cancers including melanoma. Published data indicates that CD44, perlecan, chondroitin sulfate and glypican 1 can directly influence the malignant potential of melanoma tumors; however, to date no HSPG-targeting agents have been evaluated as agents for diagnosis or prognostication. We have recently discovered a novel class of peptides that recognize hypersulfated HSPG associated with amyloid-disease, that in our preliminary studies are also capable of selectively binding to B16F10 melanoma pulmonary and nodal tumors *in vivo* and in formalin-fixed paraffin-embedded tissue sections. We will further validate this novel extracellular biomarker associated with melanoma using imaging techniques to ascertain its biodistribution primarily in mice. In addition we will expand the imaging studies to dogs with metastatic melanoma, a recognized surrogate model of the human disease [30-32]. Using this canine model at the early

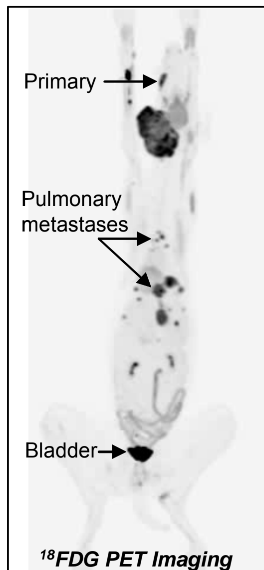

Figure 1. Imaging dogs at UTGSM and UTCVM. Melanoma was imaged using ^{18}FDG PET/CT scanning (CT data not shown) at the UTGSM.

stage of investigation will allow us to demonstrate the efficacy and limitations of the tumor-binding peptides in animals with "natural" primary tumors alone, regional disease (e.g. lymph node), pulmonary nodules or distant tissue metastases – something that arguably cannot be ascertained in current murine models of the disease. To acquire this data we will employ a newly-developed powerful comparative tri-modality imaging protocol that will use FDG-PET/CT imaging as well as gamma scintigraphy in the same subject, in order to compare the biodistribution of the peptides with known sites of hypermetabolic tumor growth. (Fig. 1 shows an example of PET imaging of metastatic melanoma in dogs using FDG as the tracer. Performed at UTGSM). This powerful dual energy, dual time imaging technique will yield quantitative comparable imaging data using individual animals.

In addition to this *in vivo* validation of the biomarker-peptide interaction we propose to use the same peptides as reagents for staining tissue sections with melanoma and assess their ability to discern tumor lesions in dogs and in human samples. We have shown that the binding of the peptides to the target in mouse tissue sections is of sufficiently high affinity that the peptide can be used as a histological stain (Fig. 4 below). The use of peptides, rather than scFv or antibodies as tissue staining reagents is a novel approach. In addition, the ability to stain tissue sections with the same reagent that, when appropriately radiolabeled, can be used for *in vivo* imaging will allow flexibility in assaying this biomarker in cases when biopsy material is not available or vice versa when access to clinical imaging platforms are not possible (this is somewhat analogous to the use of Her2/neu-reactive reagents that are used for tissue staining [33] and *in vivo* imaging of breast tumors [Reddy, #34}).

The exact biochemical structure bound by our HSPG-targeting peptides on melanoma cells has not been determined; however, as evidenced by our preliminary data (see below) this interaction can be detected and visualized specifically by routine imaging techniques and histochemistry. Quantitation of the HSPG biomarker will provide heretofore unavailable information on the phenotype of the tumor with the prospect of being able to detect and "measure" the metastatic potential of the tumor based on a non-invasive imaging protocol

(c) **Approach**

We have, in preliminary studies, used the B16F10 pulmonary lung colonization model in C57Bl/6 mice since the colonization of the lung by iv-injected tumor cells requires the expression of appropriate surface integrins and extra-cellular matrix proteins that favor colonization and growth within the lung tissue [13]. We have also observed B16F10 colonies within mediastinal lymph nodes adjacent to the heart. This murine model of melanoma tumor growth is well established and provides an excellent system in which to evaluate the reactivity of tumor-binding peptides *in vivo*. We have generated preliminary data using this model to assess the feasibility of this approach, namely the use of peptides that bind hypersulfated oligosaccharides associated with proteoglycans (a novel biomarker), to target and thereby detect the presence of melanoma tumor cells. In addition, we have developed quantitative assays that can be used to assess the efficacy of the peptide-tumor interaction.

1) *Co-localization of HSPG-reactive peptides with B16F10 melanoma pulmonary colonies using SEPCT/CT imaging*: Lung colonies were established in C57Bl6 mice by iv injection in the tail vein, of 7.5×10^4 B16F10 tumor cells. After 17-21 d, when the tumors were known to be > 1 mm in diameter, the mice were administered ~ 150 µCi of iodine-125 (^{125}I)-labeled peptide and 2 h thereafter were euthanized by isoflurane inhalation overdose and the single photon emission tomographic

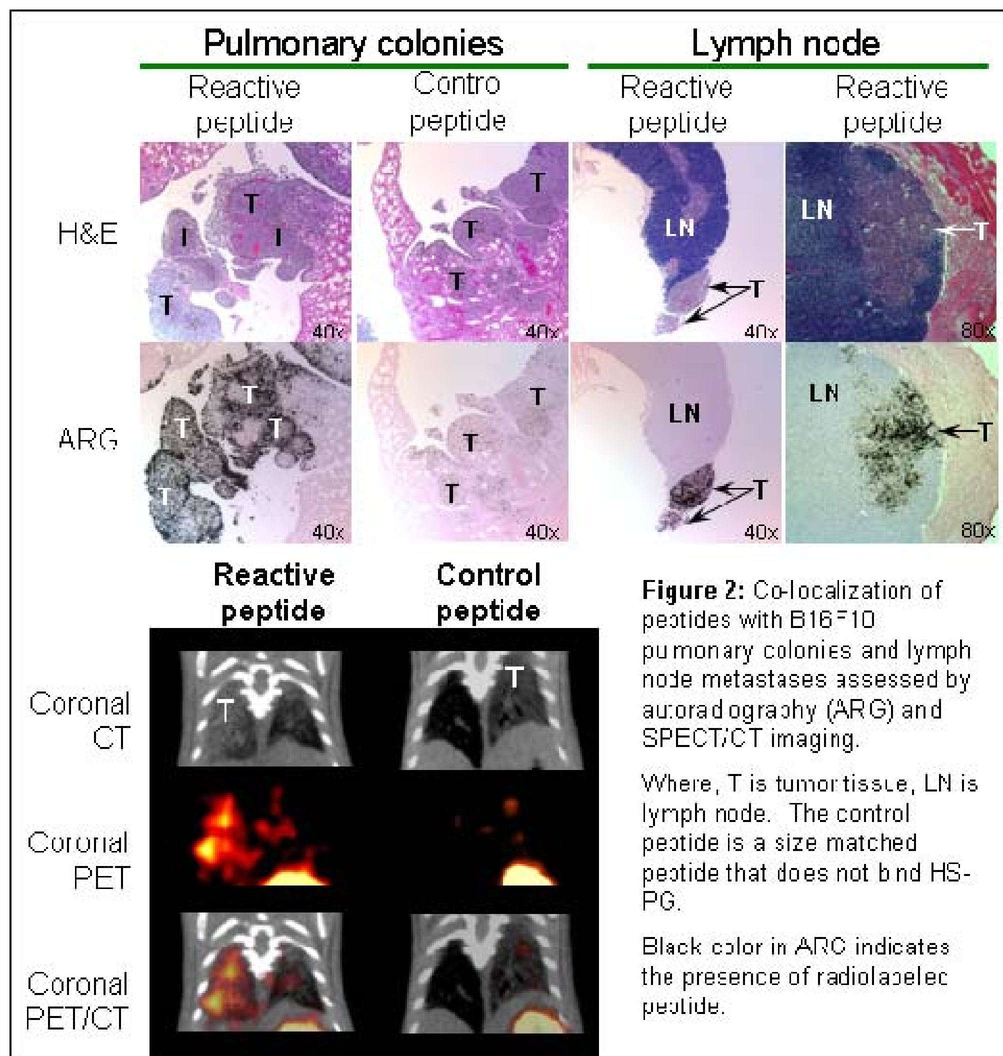

Figure 2: Co-localization of peptides with B16F10 pulmonary colonies and lymph node metastases assessed by autoradiography (ARG) and SPECT/CT imaging.

Where, T is tumor tissue, LN is lymph node. The control peptide is a size matched peptide that does not bind HS-PG.

Black color in ARG indicates the presence of radiolabeled peptide.

(SPECT) and x-ray tomographic (CT) images were acquired by using an Inveon Trimodality small animal imaging system (Siemens Preclinical Solutions, Knoxville, TN). After the image data were acquired a necropsy was performed and the heart-lung block was harvested, washed and a tumor count and tumor burden estimated by counting the number of surface-visible tumors on all 5 lobes, and estimating the burden (accounting for tumor number as well as size) on a 0 – 5+ scale, respectively. Tissues were then fixed in formalin and tissue sections prepared for autoradiographs and histochemical analyses.

Tumor uptake of HSPG-reactive peptides was evidenced in the SPECT images in lung lobes where there was CT-confirmed tumor growth (Fig. 2). The specific nature of this interaction was demonstrated using a control (inactive) size-matched peptide which did not co-localize with the tumors (Fig. 2). Autoradiography was used to confirm that the radiolabeled peptide distribution was limited to the tumor cells and not associated with the healthy lung tissue (Fig. 2). Notably, in mice where B16F10 tumor metastases were observed in sentinel lymph node tissue the ^{125}I-labeled peptide was observed labeling the tumor mass specifically and at relatively high density (Fig. 2).

These preliminary data using a representative peptide from a panel of reagents that we have generated, each with distinct physical and electrochemical properties, demonstrate the feasibility of our approach. Furthermore, these data show we have the tools capable of discerning quantitatively the binding of this peptide to HSPGs expressed by melanoma tumors *in vivo*.

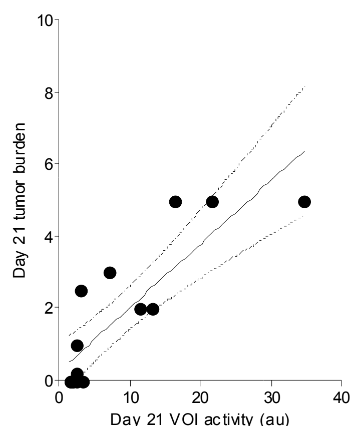

Figure 3. Correlation between ^{18}FLT PET imaging of tumor burden, at day 21 pi.

2) *Imaging tumor load with ^{18}F-fluoro-L-thymidine by PET/CT correlates with measurement of tumor load and tumor*. To assess the "true" distribution of B16F10 melanoma colonies in the C57Bl6 mice we have imaged the DNA-replication tracer ^{18}F-fluoro-L-thymidine (^{18}FLT) by using positron emission tomography (PET). This tracer was used instead of the more commonly employed and widely available ^{18}FDG for this study because it does not accumulate in the myocardium, unlike ^{18}FDG. Therefore the images of pulmonary tumors were not compromised by "spillover" from the heart. We have demonstrated that ^{18}FLT is avidly recruited by the B16F10 lung colonies and furthermore, that uptake of ^{18}FLT correlates linearly with tumor load as measured by

tumor burden (corr. coeff. = 0.84; $P < 8e^{-5}$, Fig. 3). We can therefore quantify lung tumor load non-invasively using PET/CT imaging of ^{18}FLT and this technique can be used to compare the uptake of HS-PG-reactive peptides with ^{18}FLT in the mouse lung.

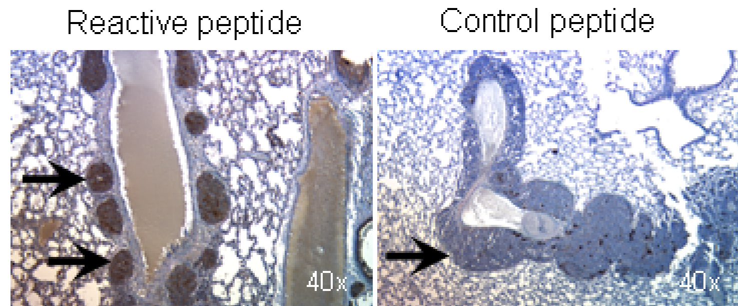

Figure 4. Histochemical staining of B16F10 pulmonary colonies (arrows) in formalin-fixed paraffin embedded tissue. The control peptide did not stain the tumors.

3) *Tissue staining of melanoma tumors using HS-PG-reactive peptides*: The final technique that we have established is the use of biotinylated HS-PG-reactive peptides for detecting tumor in tissue sections by using light or fluorescence microscopy. We will employ this technique to determine the correlation between positive "peptido-reactivity" (as opposed to immunoreactivity where antibodies are used to stain tissues) and stage of melanoma growth (*see below for details*). We will also use this method to extend our findings in mice to HSPG expression in dog and human melanoma tissue. In preliminary studies we have stained tumor tissue in formalin-fixed paraffin-embedded samples of tumor-laden mouse lung tissue. Tumor cells expressing the biomarker were readily visualized using biotinylated peptide as evidenced by the brown coloration not seen in healthy tissue (Fig. 4). We propose herein to survey the various peptides in our panel and establish, using murine, canine and human melanoma-containing tissues, the relationship between the expression of the biomarker recognized by HS-PG-peptides and tumor growth stage.

The goals of this proposal are therefore to validate and expand our preliminary findings that support the hypothesis that melanoma tumors express hypersulfated proteoglycans (either as membrane-associated cell surface proteins or within the extra-cellular matrix) which are not found in healthy tissues. We will use specific peptides as imaging probes to quantify this biomarker to provide enhanced diagnosis and prognostication. We have identified a panel of peptides that bind HS-PG and it is now our goal to assess the utility of these reagents for imaging primary and metastatic melanoma tumors *in vivo* and for detecting tumor within histological tissue sections *ex vivo*.

Aim 1:

We have designed a panel of ten, ~ 30 amino acid, basic peptides (pI > 9) that we have shown have the ability to bind HS-PG using an in vitro binding assay. We will prepare ^{125}I-labeled versions of each peptide and evaluate their ability to bind B16F10 lung colonies and lymph node metastases in mice by using small animal SPECT/CT imaging, biodistribution measurements and quantitative micro-autoradiography. Our goal is to identify the most efficacious peptides for tumor binding based on the criteria defined below. Each peptide (~ 50 - 100 µg) will be radioiodinated with ^{125}I (1.5 mCi) using chloramine T and purified by gel filtration [34]. The radiopurity of the pooled radioiodinated peptide will be assessed by performing non-reducing SDS poly acrylamide gel electrophoresis followed by phosphor imaging to demonstrate >90 radiopurity. We will also confirm retention of peptide bioactivity using the *in vitro* binding assay. The murine tumor model will use female 8-12 wk old C57Bl/6 that are injected iv with 75,000 B16F10 cells suspended in 200 µL sterile phosphate buffered saline. At 21 d pi, 3 tumor-bearing mice will receive iv, ~ 200 µCi (10 µg) of peptide in ~ 200 µL of sterile PBS. The mice will be sacrificed at 4 hr by isoflurane inhalation overdose, before acquiring high-resolution SPECT/CT images [34, 35]. At necropsy, appropriate tissues will be harvested and the tissue-associated radioactivity measured using a gamma counter – the resulting data will be expressed as % injected dose per gram tissue (%ID/g). We will also count lung colonies and estimate total tumor burden visually. Portions of each organ will be formalin-fixed, paraffin-embedded and sectioned for histochemical and autoradiographic analysis [36]. The quantitative uptake of each peptide in tumor will be assessed by:

i) Comparing %ID/g for the lung tissue relative to muscle (a control tissue)

ii) Quantifying the activity in each heart lung block volume of interest from the SPECT images [35].

iii) Measuring the uptake of peptide in tumor as evidenced in the autoradiographs – the peptide density will be quantified by digital analysis of microscopic images.

The most efficacious peptide, based on these 3 uptake metrics will be used in a comparative study using FDG and FLT in the lung tumor mouse model. For these studies, tumored mice at 21 d pi will receive iv ~ 200 µCi of FDG or FLT in addition to 200 µCi of ^{125}I-labeled peptide in a total volume of 200µL. To avoid lung motion aberrations in the scans, the mice will be euthanized 2 h pi and placed on a disposable cardboard imaging palette for PET/CT imaging (Inveon Trimodality system). The mice will then be frozen, on the imaging palette, at -20°C for 24 h for decay of F-18 and then SPECT/CT images of the remaining I-125 acquired. The PET, SPECT and CT images will be co-registered and the biodistribution of each tracer assessed and compared by visual inspection of the images. These studies will be used to confirm that the peptides are binding to sites of known tumor growth *in vivo* based on FLT and FDG uptake.

AIM 2: In this study we seek to assess the validity and limitations of using biotinylated HS-PG –reactive peptides as reagents for detecting the presence of human and canine melanoma tumor in formalin fixed paraffin embedded tissue sections. For this we will evaluate the 3 most efficacious tumor binding peptides described above for their ability to discern melanoma from healthy tissues. We will study the binding by quantitative histochemistry of each peptide to primary and metastatic melanoma lesions, as well as healthy tissue samples. The stained melanoma tissue sections (human samples will be obtained from a repository at the Moffitt Cancer Center, FL and canine samples will come from Dr. R. Donnell, co-investigator on this project) as well as those from healthy tissues (to assess background reactivity) will be analyzed by quantifying the staining seen in low-power (5× obj.) microscope images using image segmentation tools [37]. Our hypothesis, that the peptides can discern melanoma tumor within a tissue section will be tested in this initial pilot study by determining the specificity and sensitivity of the staining using 20 tissue samples.

Aim 3: (Yr 2), Based on the peptide imaging data in mice we will select a single peptide for use in dogs with metastatic melanoma. Since the canine imaging studies require a ^{99m}Tc-labeled peptide, we will perform a preliminary study using the most efficacious *in vivo* targeting peptide labeled with ^{99m}Tc [38] in mice with pulmonary B16F10 colonies to assure that it retains its bioactivity using this radionuclide. The peptide, labeled by tin reduction of ^{99m}Tc (pertechnitate) coupling to an available cysteine residue will be assayed for radiochemical purity and bioactivity with the same assays used for I-125 peptide. These data will be compared with the ^{125}I-peptide data to ensure no quantitative loss in binding occurs. In the dog imaging studies we will compare the biodistribution of FDG and ^{99m}Tc-peptide in the same animal to determine whether the biomarker recognized by the peptide is expressed on all FDG-positive lesions or whether there is selective binding for example to the primary, sentinel nodes, pulmonary lesions or distant tissue metastases. After obtaining informed consent, 9 dogs diagnosed by Dr. Amy LeBlanc (PI on this proposal) with metastatic melanoma will be sedated, intubated and placed under isoflurane anesthesia before acquisition of FDG (5 mCi) PET/CT images (see Fig. 1 for example). The dogs will be recovered, and 24-36 h thereafter will be given 5 mCi of ^{99m}Tc-labeled peptide and after a further 4 h they will be sedated and planar gamma images of the peptide distribution acquired. We will compare the biodistribution of FDG and the ^{99m}Tc-peptide in the same way using 3 size-matched control hounds. The distribution of the peptide will be correlated with the FDG images and an assessment of co-localization will be made by Dr. Federica Morandi, Chair of the Dept. of Veterinary Radiology and a consultant on this application. These studies will validate the use of an HS-PG targeting peptide for the in vivo detection of melanoma and assess the selectivity of this interaction with the various lesions seen in canines with metastatic melanoma – a naturally occurring model of the disease and a recognized surrogate of the human condition.

Melanoma is a prevalent and devastating disease. The studies proposed above will yield a novel peptide reagent that targets a biomarker present on melanoma tumors, whose expression may be of great diagnostic or prognostic importance, and whose detection may enhance early diagnosis, staging, and effective patient treatment.