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TITLE: Development of Tethered Hsp90 Inhibitors Carrying Radioiodinated Probes to Specifically Discriminate and Kill Malignant Breast Tumor Cells

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14. ABSTRACT
Our work this year focused on continued development of our chemistry platform with the synthesis of a novel photo-acoustic tethered Hsp90 inhibitor. Our goal is to enable this probe for PET imaging by introducing an iodine. This probe combination will have better utility for interfacing our technology with current standard of practice mammography programs. Pharmacokinetic studies with ^{124}IHS-227, a Cy5 carrying radioiodinated tethered Hsp90 inhibitor, showed very rapid clearance following injection into tumor bearing mice. Our studies suggest that large bolus doses or infusion are required to enable effective tumor detection by PET/CT.

15. SUBJECT TERMS ^{124}IHS-227, a radio-iodinated far red fluorophore tethered to an Hsp90 inhibitor, PET -positron emission tomography

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1. Introduction

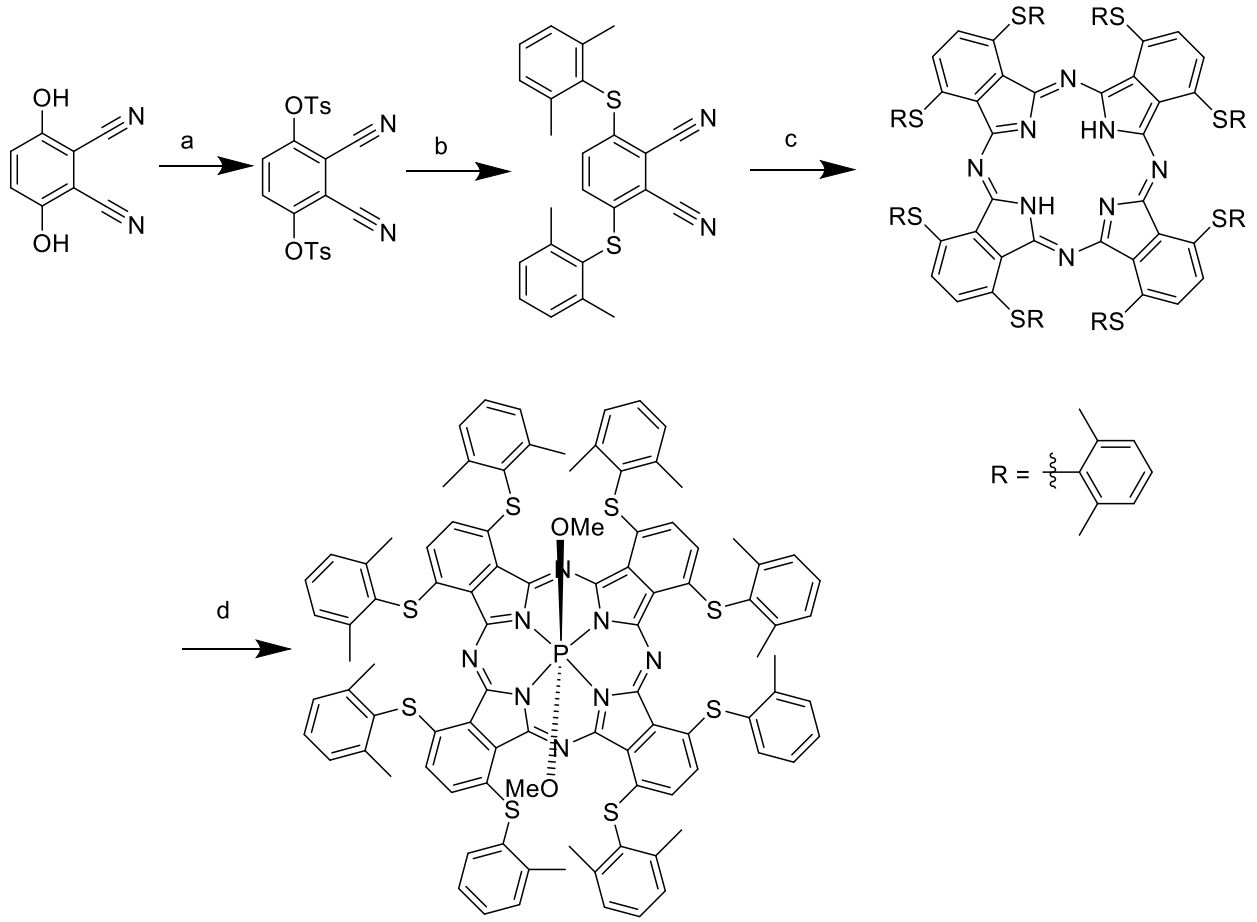
In the US, routine breast cancer screening results in over 1.6 million biopsies annually leading to the diagnosis and surgical resection of breast cancer or breast carcinoma *in situ* in over 250,000 women respectively. Unfortunately, the sensitivity but low specificity of screening has led to concerns about over treatment of indolent disease, as evidenced by the increased incidence and treatment of early stage breast cancer without a concomitant decrease in the nearly 40,000 breast cancer deaths annually. Clinical data indicate a strong link between high expression/activation of Heat shock protein 90 (Hsp90) with poor prognosis in malignant breast cancer (Cheng et al., 2012; Pick et al., 2007). Specifically, immunohistochemical analysis of breast cancer cell lines and 655 primary breast cancers (including 331 ER+ and 324 ER- tumors) showed increased Hsp90 expression in all breast cancer cell lines, and in nearly 90% of primary breast cancers (Pick et al., 2007). A recent study at our institution evaluated Hsp90 gene expression from profiles of over 4,000 breast cancer patients from 23 publically available gene expression databases, which also reported overall survival data from over 1000 patients. This study confirmed up regulated Hsp90 was associated with poor overall survival in all breast cancer subtypes including estrogen (ER) negative, HER2 negative and triple negative breast cancers (Cheng et al., 2012). Our laboratories recently developed a series of optical and iodinated tethered Hsp90 inhibitors that have exquisite selectivity *in vivo* for metastatic breast tumors expressing ectopic (cell surface) Hsp90 (Jared J. Barrott, 2013). We also discovered that ectopically expressed Hsp90 is rapidly internalized and can carry these tethered inhibitors specifically into the breast cancer cells. This work in tandem with published clinical results suggests that selective targeting of Hsp90 up regulated in malignancy may present an opportunity to not only discriminate indolent tumors from metastatic disease, but also offer a molecularly targeted radiotherapy approach for body wide tumor ablation with low normal tissue toxicity. Herein, we propose to develop a series of tethered Hsp90 inhibitors capable of selectively delivering radioiodine (^{124}I and ^{131}I) or ^{211}At to malignant tumor cells. We envisage a process in which a patient, after standard of care breast exam, is first evaluated for malignancy vs. indolent disease by positron emission tomography (PET) imaging using ^{124}I-labeled tethered inhibitors. Then, in patients with malignancies detected in high contrast to normal tissues, targeted radiotherapy would be preformed at patient-optimized doses of inhibitor labeled with the β-emitter ^{131}I or the α-emitter ^{211}At. **This is an attractive strategy for breast cancer because the same molecules can be used to not only discriminate indolent disease from metastatic, but also enables selective tumor ablation on a personalized level, potentially mitigating life altering side effects commonly associated with current chemotherapeutics or radiation strategies**.

2. Keywords

Radioiodinated, Tethered Hsp90 inhibitor, malignant breast cancer.

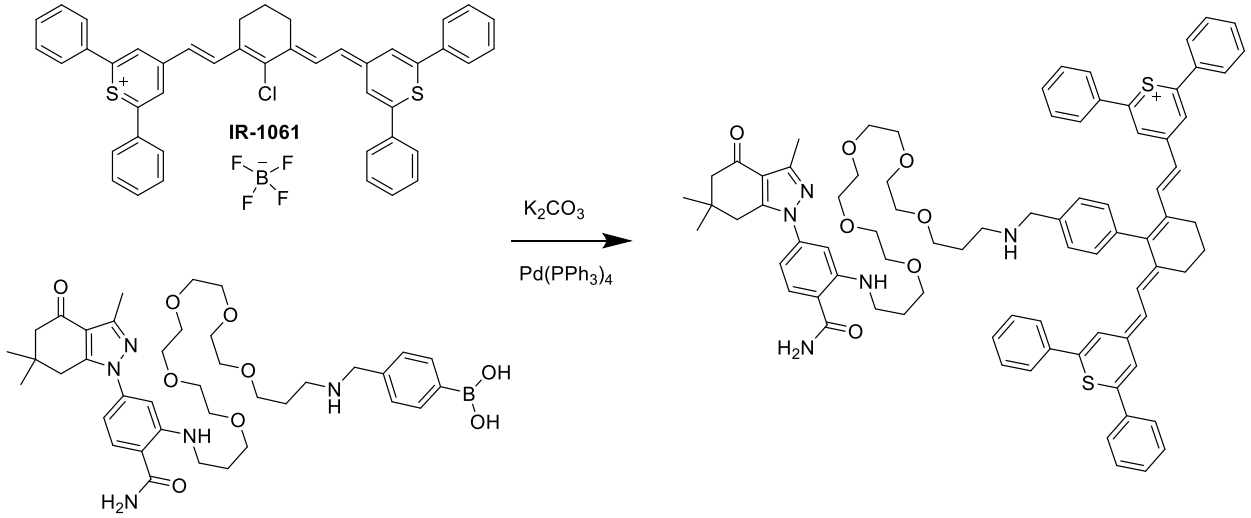
3. Accomplishments.

Development of PET enabled Photoacoustic Probe: In last year's report, we described the development of iodinated analogs of near IR probes that utilized cyanine dyes and emitted, depending on the dye, from 680 nm to 820 nm. Detection of these dyes is much more sensitive and much less prone to interference. One of the ultimate aims of these probes is to interface them with mammography and we continue to synthesize new tethered probes that will enable this process. Ideally, we would like our probes to be detectable non-invasively in the breast. One way to do this may be using photoacoustic technology. We envisage a process in which patient is administered a PET-enabled photoacoustic tethered Hsp90 inhibitor and the breast examined with an ultra-sound device (similar to that used in pregnant women to look at the developing foetus). If a mass is detected, it could be biopsied as normal, and/or the patient offered a radiolabeled version carrying non-lethal ^{124}I for whole body imaging by PET. Ultimately, if metastatic disease is detected with ^{124}I, a ^{131}I version is administered to ablate the tumor masses. Photoacoustic dyes vibrate at specific frequencies that can be detected audibly in response to light. If one can identify light wavelengths that are deeply penetrating through tissues, one can now detected our probes deep within tissues by ultrasound. Typically wavelengths in the 1000nm range are thought to be the most penetrating and optimal to produce a photoacoustic response for a given dye. In order to develop such a probe with our existing chemistry platform, our first goal was therefore to find or develop dyes which have a strong absorption near the primary emission of the Nb:YAG laser (em1000nm) and to couple these to our Hsp90 targeting ligand. Our initial work aimed at making the analogs phthalocyanines described by Kobayashi(*1*) and shown in Scheme **1**. The dyes described by Kobayashi are symmetric and have no point of attachment and are made by a cyclization of 4 subunits. Our plan was to make one subunit with an attachment point and take the statistical hit on the cyclization. The Kobajashi dye has been used in photoacoustic spectroscopy by Zhou *et.al.*(*2*). Unfortunately, the second step (b) of the synthesis never gave better than 11% in spite of numerous attempts (17 rxns.) to improve the yield. This was the same yield reported by Zhou though others have reported higher yield. Given the statistical nature of the later steps, this approach was put aside.

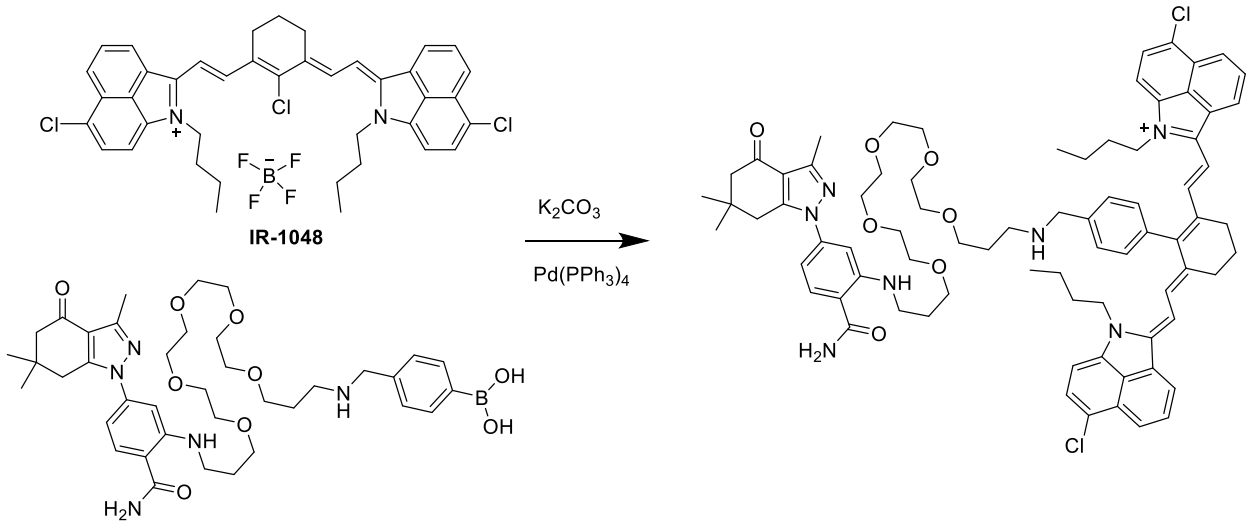


Scheme **1**. Synthesis of a Phthlocyanine dye with absorbance near 1000 nm.

A search for dyes with an absorbance around 1000 nm turned up a couple of commercially available dyes, which, theoretically, could be coupled to our probe via a Suzuki coupling much like HS-131 and HS-196. They are IR-1061 and IR-1048. We ran the standard coupling reactions as shown in Scheme **2** and **3**.

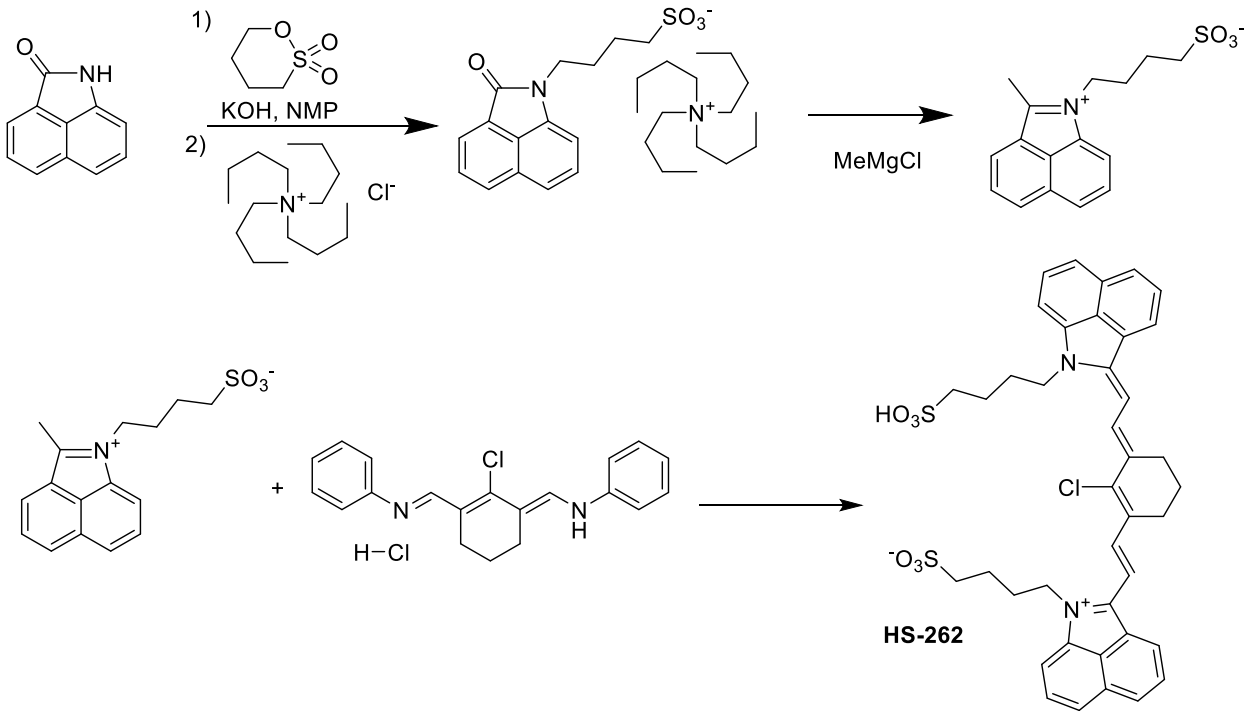


Scheme **2**.



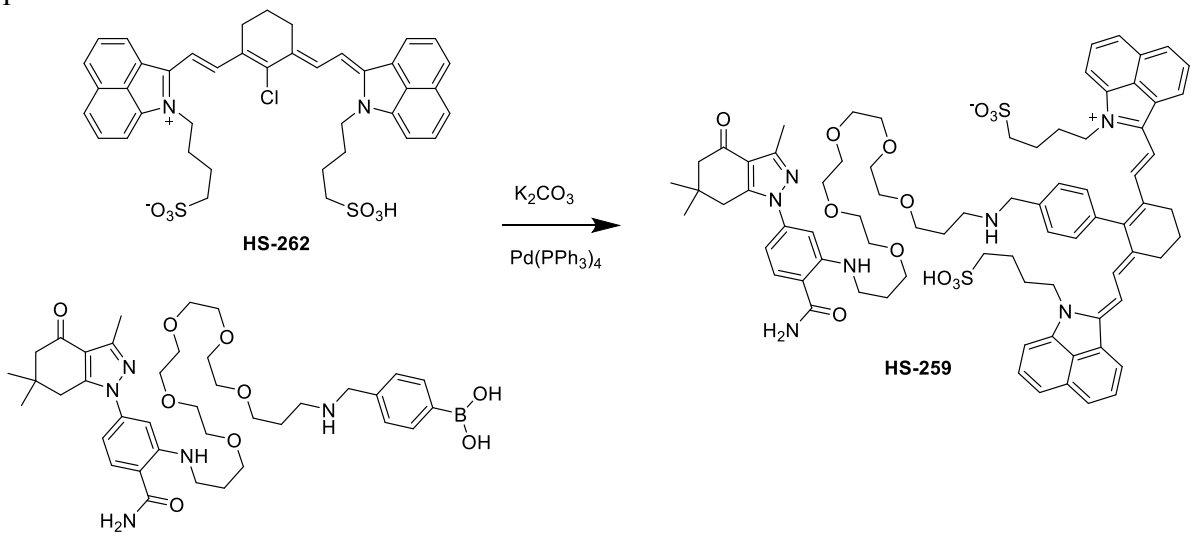
Scheme **3**.

One of the complaints about dyes that absorb around 1000 nm is that they generally suffer from poor solubility. Although, we may have made the right products as described in schemes **2** and **3**, we were unable to solubilize anything that gave spectral evidence of being the desired products. Given the similarity of dye IR-1048 to the cyanine dyes that we have used in the past, we looked for similar dyes that might have solubilizing sulfonic acids seen in the dyes used for HS-131 and HS-196. We were able to find a report of a bis-sulfonate by Henary et al.(*3*). Using their synthetic procedure, we were able to produce the desired dye which we have registered as HS-262, as shown in Scheme **4**. Because of its poor solubility, it was initially difficult to obtain pure HS-262 and so impure material was used for the final reaction.



Scheme **4**. Synthesis of HS-262.

We were able to couple HS-262 to our boronate linked ligand to the dye HS-262, using the standard Suzuki conditions, to give the desired probe HS-259 with a yield of around 7% after numerous purifications.



We are currently working to improve the yield of the reaction and develop better ways of working with the product. HS-259 absorbs at 1005.5 nm which is still not optimal for the Nd:YAG laser whose primary emission is at 1064 nm. Further substitution of HS-259, perhaps by sulfonylation, might give a more favorable absorbance with better solubility. Once we have tested the bioavailability of our newly synthesized photoacoustic probes in cells and animals we will proceed to the synthesis of PET Enabled versions.

Studies with ^{124}IHS 227. In the previous report we described the synthesis of HS-227, PET enabled version of HS-131. HS-227 is designed to serve as an alternate molecule to HS-113, in the event that the later should fail in animals studies and subsequent preclinical development studies. HS-113 also shows rapid clearance in vivo with primary route of elimination via the biliary system. Following synthesis of the stannylated versions of HS-277 we worked again with the UNC small animal imaging facility to produce the ^{124}I version. We then employed their services to study the pharmacokinetics of the molecule in mice bearing flank breast tumors. The images below showed very little evidence of tumor uptake into the flank tumors by PET imaging alone over time in contrast to ^{124}IHS-113 (Fig.1). Again the major route of elimination appears to be via the biliary system. These findings were surprising since we had shown by fluorescence that HS-227 entered

HER2+ and triple negative cell lines in culture. Several reasons for this outcome include rapid elimination or metabolism of the molecule. These two possibilities are currently being investigated. **NOTE. No DOD or Federal funds were used for any of the animal studies carried out at UNC**. Private funds were used to fund the animal studies. DOD funds were used to synthesize HS-227 and produce the stannylated precursor molecules.

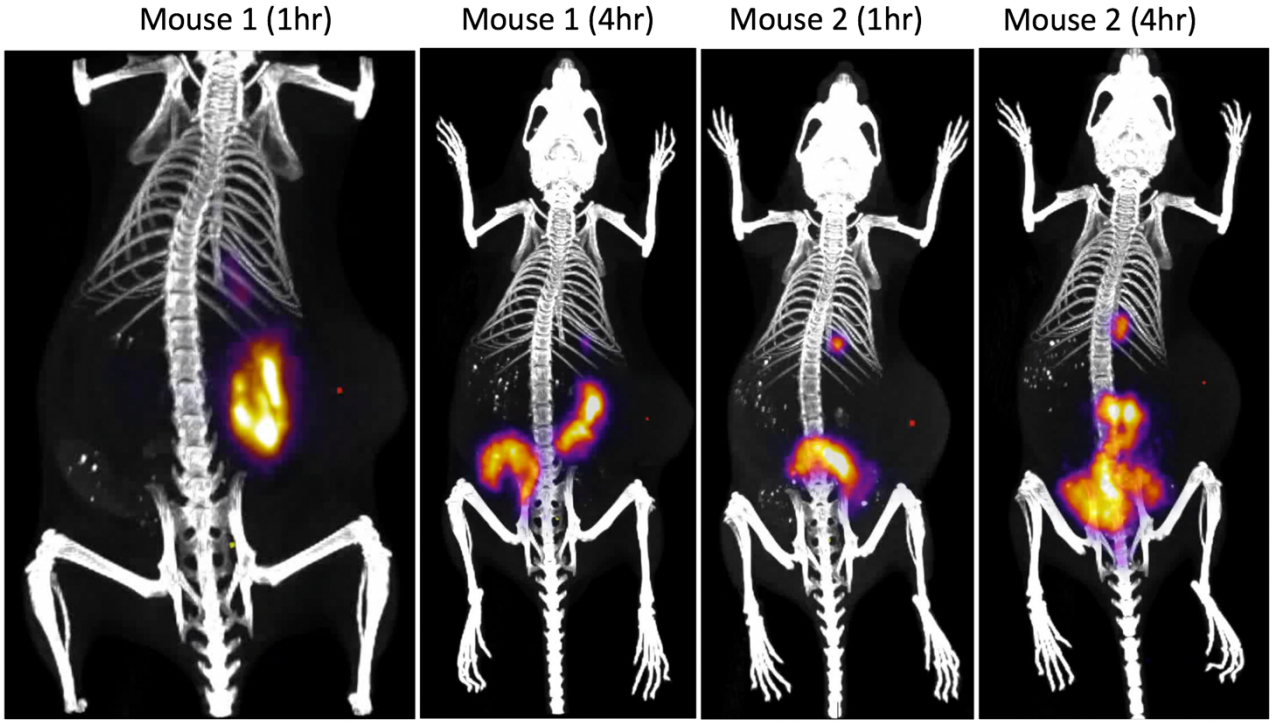


Figure 1. PET study of tumor bearing mice injected with ^{124}IHS-227 following probe distribution over time.

The routes of elimination observed by PET/CT were confirmed after carrying out analysis post-mortem of tissues isolated from the treated animals (Figure 3).

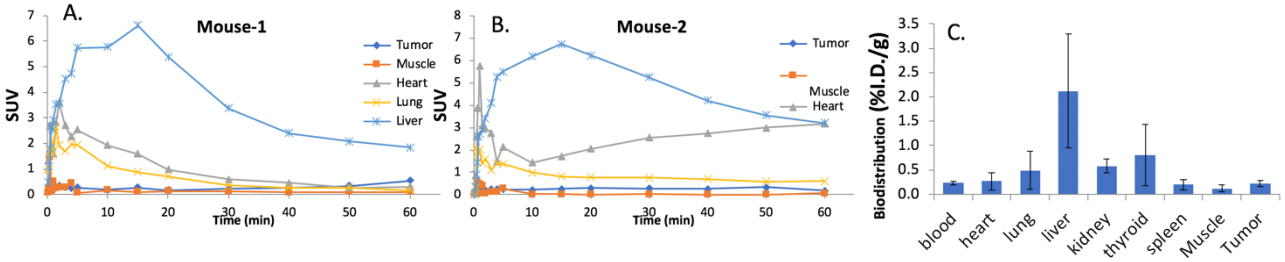


Figure 3. The Biodistribution of ^{124}HS-227 as measured by PET imaging and by radioactive tissue content. In A and B, tissues identified and quantified by PT/CT imaging. In (**C**) tissues were collected at 4hr postmortem and counted in a scintillation counter.

4. Impact:

Our studies indicate that both our original PET enabled probe HS-113 and HS-227 can be reliably and reproducibly synthesized both in non-radioactive and radio-iodinated forms. When injected into animals as radiotracers both are rapidly cleared to the extent that in one case (^{124}IHS-227) we observed little evidence of selective tumor uptake, in contrast with ^{124}IHS-113. This problem maybe readily solved by using higher pharmacological doses or through slow iv infusion. Both probes are cleared via the hepatobiliary system and these is deemed to be desirable for a PET agent that subsequently may be developed into a targeted therapeutic. Ideal PET agents are eliminated quickly to minimize unnecessary radiation exposure as well as maximize contrast within the targeted tumor cells. Elimination into the gut may also reduce the risk of radiation exposure due to unbound probe because the this will mix with digestive contents of the intestinal track. If used clinically, once the unbound probe has entered the bowl, this could be efficiently flushed with an enema to further reduce exposure and enable tumors within the gut wall or

nearby, to be detected by PET imaging.

5. Changes/Problems.

Changes: Collaboration with the Ramanujam lab. Although not a part of the original SOW, we synthesized the molecule HS-27 (a non-iodinated active analog of HS-113) using DOD funds associated with the proposal. We then supplied this molecule to the Ramanujam lab to ask a fundamental key question; *does surface Hsp90 is expressed in actual human breast tumors*? Dr Nimmi Ramanujam (Duke) has an IRB approved protocol to collect breast tumor biopsies from patients to carry out metabolic studies across all breast tumor subtypes. We provided Nimmi with HS-27 and she obtained a modification of her IRB to test any collected biopsies for probe uptake. Specifically, needle biopsies taken from fresh breast cancer resections were immediately mixed with HS27 (a version of HS113 non-radioactive) for 1 minute. The biopsy was washed to remove excess probe, then examined by a fluorescent microscope to quantitate probe uptake. Figure 2 shows selective uptake of HS27 into all 3 major breast cancer molecular subtypes (ER+, triple negative and HER2+) with low uptake into biopsies from non-malignant masses, and no uptake in normal breast tissue (provided from reduction mammoplasty). Although this study is ongoing, these data provide strong evidence that the most aggressive forms of human breast cancer do indeed express surface Hsp90, and that this can be detected using our probes. The goal is to extend this approach to interface with mammography to improve detection of aggressive disease by Hsp90-PET imaging, paving the way to non-invasively ablate tumors, including macro- and micro-metastatic sites with a ^{131}I carrying version.

Benign Biopsies (10)	
Fibroadenoma	3
Fat necrosis	2
Fibrosis	1
Tubular adenoma	1
Atypical ductal hyperplasia	1
Cystic tissue	1
Benign near cancer	1
Mammoplasty Biopsies (6)	
Tumor Biopsies (27)	
ER+	22 (81%)
Her2+	5 (19%)
TNBC	4 (15%)
Demographics	
Average age (range)	55 (25-79)
BMI	30.4
Mammography Breast Density	
Fatty	4
Scattered Fibroglandular	13
Heterogeneous Density	15
Extremely Dense	5
BIRADS Score	
4a	4
4b	7
4c	11
5	15

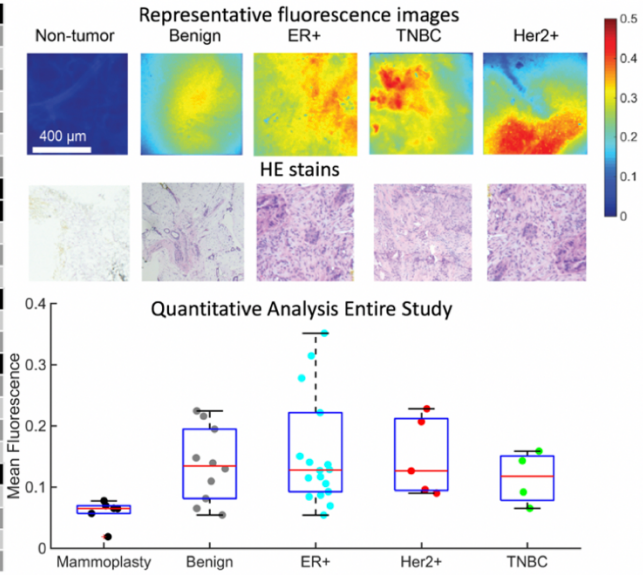


Figure 2. HS27 uptake into breast tumor needle biopsies (data provided Brian Crouch, Global Women's Health Technologies Center (PI Nimmi Ramanujam)

Problems: Both HS-113 and HS-227 are rapidly eliminated after a single bolus dose, most likely at the detriment of tumor uptake. Rapid elimination for any PET agent is in fact desirable, but clearly we need to establish conditions where the probes can be maintained in circulation for sufficient time to ensure tumor exposure. This can be achieved in two ways 1) a large bolus dose at pharmacological levels rather than tracer. 2) IV infusion over a fixed time. The later scenario is attractive because it would allow blood levels to be maintained at a constant for a fixed time, and then once stopped, the free unbound probe would be cleared rapidly. Our goal is to first explore these approaches with non-radiolabeled versions and then proceed to studies with radio-iodinated versions if we can attract additional funding.

6. Products

HS-227 was synthesized in three formats, non-radio iodinated, stannylated (Sn substituted) and as a radio-iodinated form, ^{124}IHS-227. We synthesized HS-27 for new work with Dr

Ramanujam. These studies indicate that this probe could be used in clinical setting as a diagnostic tool with “live” needle biopsies to identify early metastatic disease.

7. Participants & Other Collaborating Organizations……

Grants.gov	Requested
ID Number	GRANT11490422
Principal Investigator	Michael Zalutsky
Performing Organization	Duke University
Contracting Organization	Duke University
Partner	
Budget	$549,500
Direct Costs	$350,000
Indirect Costs	$199,500

8. Special Reporting Requirements

none

9. Appendices

none

10.References:

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(2) Zhou, Y., Wang, D. P., Zhang, Y. M., Chitgupi, U., Geng, J. M., Wang, Y. H., Zhang, Y. Z., Cook, T. R., Xia, J., and Lovell, J. F. (2016) A Phosphorus Phthalocyanine Formulation with Intense Absorbance at 1000 nm for Deep Optical Imaging. *Theranostics 6*, 688-697.

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