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

PRINCIPAL INVESTIGATOR:

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CONTRACTING ORGANIZATION:
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14. ABSTRACT
We have proposed to develop tethered Hsp90 inhibitor capable of carrying various radioactive iodine isotopes for early detection and ablation of metastatic breast cancers. These probes specifically target a surface form of heat shock protein 90 (eHsp90) that is expressed on malignant cells and internalized. In this report we describe our chemistry efforts to synthesize a non-radioactive tethered Hsp90 inhibitor and methods developed for stannylation of the molecule such that it can be effectively radioiodinated with either ^{131}I or ^{124}I. Two paths of synthesis were developed and optimized. A standard operating procedure was developed and then adapted and modified to provide workable protocols for radioiodination. An inactive control molecule was also developed, which can also be labeled with radioiodine to permit determination of specificity of radio-ligand localization. These molecules will be tested as imaging agents in mouse models of breast cancer.

15. SUBJECT TERMS
Radiodination, tethered Hsp90 inhibitor, malignant breast tumor, ectopic Hsp90

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Table of Contents

Page

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. Specifically, immunohistochemical analysis of breast cancer cell lines and primary breast cancers demonstrated increased Hsp90 expression in all breast cancer cell lines, and in nearly 90% of primary breast cancers. Moreover, a recent study at our institution evaluated Hsp90 gene expression from profiles of over 4,000 breast cancer patients from publicly available gene expression databases that also reported overall survival data from over 1000 patients. This study confirmed that up regulated Hsp90 was associated with poor overall survival in all breast cancer subtypes including estrogen-negative, HER2-negative and triple- negative breast cancers.

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. We discovered that this ectopically expressed Hsp90 is rapidly internalized and can carry these tethered inhibitors specifically into breast cancer cells. This work, in combination with the published clinical results described in the preceding paragraph, 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 systemic tumor ablation potentially with low normal tissue toxicity. In this project, we proposed to develop a series of tethered Hsp90 inhibitors capable of selectively delivering radioiodine (^{124}I and ^{131}I) or ^{211}At to malignant breast cancer cell populations. With these tools in hand, 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 an ^{124}I-labeled tethered inhibitor. Then, in patients with malignancies detected in high contrast to normal tissues, targeted radiotherapy would be performed at patient-optimized doses (based on the PET scan) of inhibitor labeled with the β-emitter ^{131}I or the α-emitter ^{211}At. ***This is an attractive strategy for breast cancer management*** *because the same molecules can be used to not only discriminate indolent disease from metastatic, but also for selective tumor ablation on a personalized level, potentially mitigating life altering side effects commonly associated with current chemotherapeutics or radiation strategies.*

2. Key Words.

Hsp90, PET, ^{124}I, ^{131}I, ^{211}At, targeted radiotherapy, tethered Hsp90 inhibitor, breast cancer

3. Accomplishments.

The overall objective of this research project, in concert with the partnering PI Haystead project, is to develop tethered Hsp90 inhibitors (T90i) that can be utilized for duel imaging modality detection of breast cancers by PET and optical imaging, such that they can be readily interfaced with current mammography practice. With this goal in mind, we extended our existing tethered Hsp90 synthetic platform to generate and evaluate T90i molecules that could first be used for histology with the nonradioactive iodine-containing analogue, and then for noninvasive whole body PET imaging with the same molecule in which the nonradioactive iodine atom is replaced by ^{124}I, and ultimately, in the therapeutic step, replaced by the beta-particle emitter ^{131}I for selective tumor ablation. In this so-called “see and cure” approach, if fluorescence microscopy of biopsy slides from a breast cancer patient reveals uptake of the probe, and this concurs with the pathology diagnosis, the patient would be offered the^{124}I-labeled version for whole body PET imaging to identify evidence of disease progression. If warranted by results, this could then be followed by targeted radiotherapy with the ^{131}I-labeled analogue. In this scenario, the physicochemical properties of the molecule are the same at all stages, just the isotope changes. This is a major advantage for the clinical development pathway because the initial safety studies (GMP/GLP etc) prior to PET imaging can be carried out with the nonradioactive material. A variety of synthetic approaches were developed and evaluated eventually leading to a synthetic pathway appropriate for a series of fluorescent probe structures also carrying iodine that could subsequently be converted into stannylated precursors for radioiodination. Not surprisingly, during the development of these probes, we discovered that placement of iodine within the ligand binding region inactivated the molecule completely and that placement of the iodine distally and close to the fluorophore was essential to retain activity, both *in vitro* and *in vivo*. The original narrative outlined the synthetic pathway for two T90i , HS-113 and HS-227, that were generated. Complete synthetic pathways for all the T90i molecules containing an iodine (radioactive or not radioactive) are described in recently issued US and worldwide patents (US9,738,643 B2, US 2015/0191471 A1, WO2014/025395 A1).

In collaboration with the Haystead laboratory, we then developed a standard operating procedure for ^{124}I radioiodination of the tin precursors of HS-113 and HS-227. Recent published work with non-radioactive fluorescent versions has enabled us to define the molecular mechanism by which PET imaging-amenable T90i recognize ectopic Hsp90 (eHsp90) when it is expressed on the surface of

multiple malignant breast tumor lines (*Barrott et al. Cell Chem. Biol. 2013;20(9):1-11; Crowe et al. ACS Chem. Biol. 2017, 12, 1047−1055*). Additionally, independent studies by the Ramanujam lab demonstrated direct uptake of a non-radioactive analog of HS-113 into fresh needle biopsies obtained from women undergoing lumpectomy and diagnosed with either HER2+, ER+ or triple negative breast cancer (*Crouch et al. Sci Rep. 2017;7(1):17487*). These studies are important because they provide evidence that aggressive forms of breast cancer selectively express eHsp90 in humans. Collectively, these studies demonstrate that a T90i carrying a fluorescent probe have great utility for preclinical mechanistic studies as they can be detected by confocal microscopy, histological analysis and isolation of eHsp90 positive tumor cells by flow cytometry. In collaboration with the Haystead lab and the Small Animal Imaging (SAI) core and mouse phase 1 unit (MP1U) at UNC-Lineberger Comprehensive Cancer Center at the University of North Carolina, we tested our ability to image [^{124}I]HS-113 by PET in the MMTV mouse model of HER2-positive breast cancer. MMTV mice produce spontaneous primary and secondary tumors that are thought to mimic the cellular heterogeneity of tumors observed in human disease, which are often a mixture of premalignant, malignant, normal stromal tissue and benign cells. The emergence of primary tumors is largely confined to mammary tissue, but secondary metastatic deposits can be generated 60-70% of the time. Figure 1 highlights two separate studies in which we injected [^{124}I]HS-113 into MMTV mice bearing tumors (No DOD funds were used for these animal studies) . In both examples, we observed discrete probe accumulation in secondary tumor masses consistent with prior published work

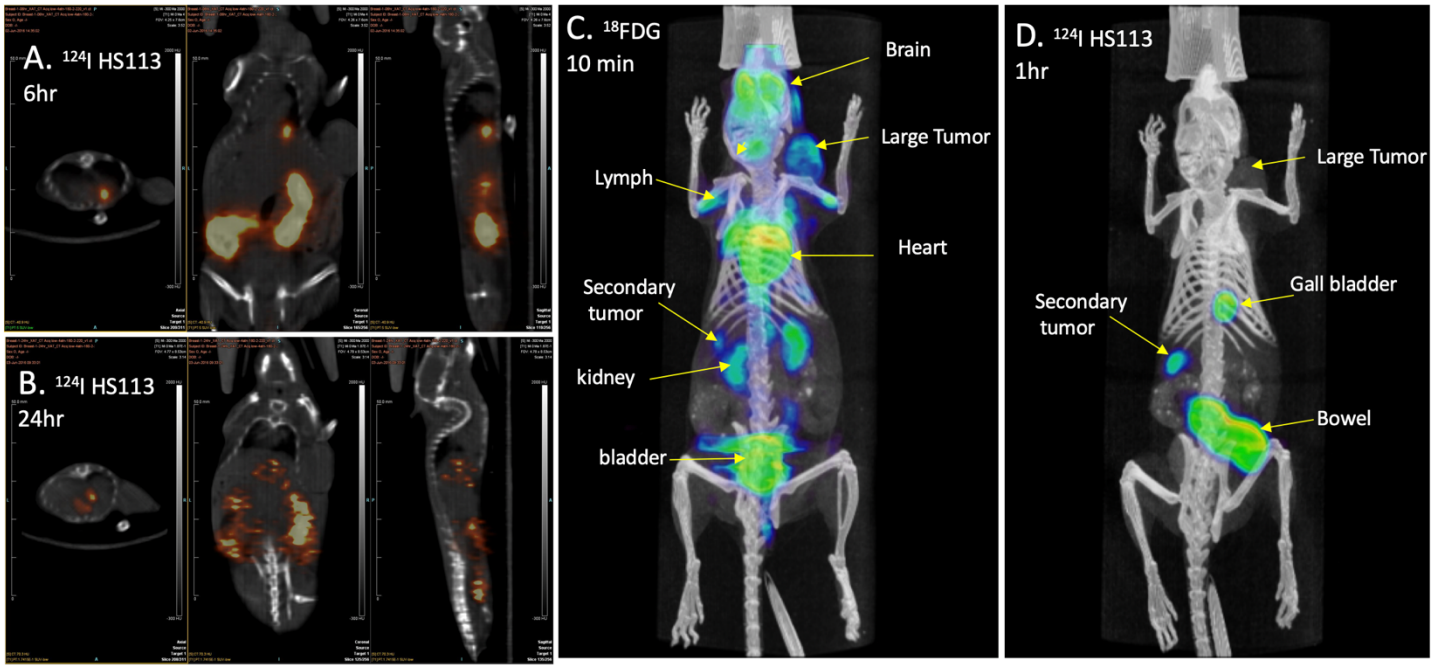

Figure 1. PET/CT images showing that ^{124}IHS-113 detects primary and secondary tumor masses in mouse models of HER2+ breast cancer. (**A**) and (**B**) shows a time course of a single mouse injected with a trace amount ^{124}IHS-113 and imaged by PET/CT at two time points. A mass is detected at 6 hours in the lung. The majority of radioactivity is cleared via the bowl by 24 hrs. In (**C**) an MMTV mouse bearing a primary tumor (neck region) was injected with ^{18}FDG (5mM) and imaged by PET/CT. Uptake in all major organs are detected along with the primary tumor mass and a secondary mass in the mid region. In (**D**) the same animal was injected 24 hrs later with 1nmol of ^{124}I HS-113 and imaged by PET CT at 1 hr. The images shows discrete uptake in the secondary metastatic mass with more discrete uptake in the primary mass. No other organs exhibited uptake in contrast to ^{18}FDG, illustrating the selectivity of ^{124}IHS-113. As with the example in (**A**) and (**B**), ^{124}HS-113 was eliminated primarily through the bowl via the biliary system.

suggesting a correlation between expression of eHsp90 and a metastatic phenotype. Figure 1C and 1D starkly highlight the specificity of [^{124}I]HS -113 relative to [^{18}F]FDG, the later showing broad uptake in metabolically active tissues such as kidney, heart and brain. These promising results also discriminate our PET-amenable T90i compound from that of a radioiodinated form of the Hsp90 inhibitor PU-H71 reported by Larson and colleagues at Memorial Sloan Kettering. In their clinical PET/CT study, following injection of radioiodinated PU-H71, the drug showed non-specific whole-body distribution, consistent with a diffusible drug and ubiquitous expression of Hsp90 in most normal cell populations. However, after 48 h, as the radiotracer cleared systemically, tumor-specific contrast emerged. Although radiolabeled PU-H71 might have value as diagnostic tool, its utility as a platform for developing a therapeutic agent is limited because of its broad non-targeted distribution in normal tissues.

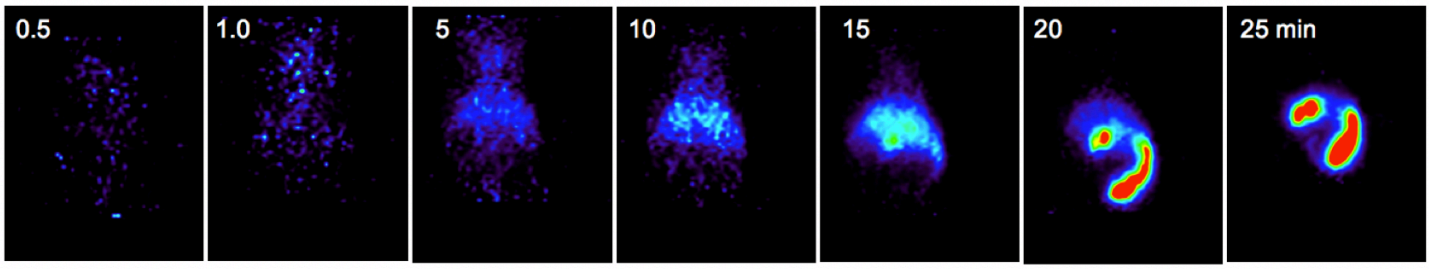

Figure 2. Time lapse sequence following the real time elimination of ^{124}IHS-113 by PET imaging in a mouse. The animal was injected with a tracer amount of ^{124}HS-113 and the signal followed continuously over 30 min. Images show the free probe rapidly collecting in the liver and condensing into the biliary ductal network within 10 minutes, followed by concentration into the gall bladder (15 min). By 20 minutes the probe is emptied into the small intestine. Images are snap shots taken from a movie.

One of the most striking features of our studies with [^{124}I]HS-113 is its rapid and exclusive clearance through the biliary system (Figure 2). Other Ti90s that we have examined such as HS-196 are primarily eliminated through the kidneys. In the proposed Aims, we will compare the PK properties of [^{124}I]HS-113 which carries a fluorescein moiety, with [^{124}I]HS-227, which carries a Cy5 far red dye. Our goal is to have a PET-amenable T90i that can be micro dosed with serum clearance times of 1-2 hrs. We also prefer to have the clearance through the biliary system and into the bowel. Elimination of the radiolabeled versions via the intestine may be desirable since the intestinal contents are likely to reduce irradiation of the gut wall. Rapid clearance via the liver is also desirable since this also limits any potential for secondary tissue damage if the radiolabeled T90i persisted for some time in the circulation and were more slowly eliminated.

4. Impact.

These imaging studies suggest that addition of the benzyl iodide alone dramatically alters the rate and route of probe clearance compared to prior fluorophore-tethered inhibitors. Likely explanations

include that either the molecule is rapidly metabolized releasing the iodinated prosthetic or HS-113 interacts in some manner with the hepatobiliary network via the benzyl iodide group.

5. Changes/Problems

As noted previously, it has been difficult to identify structures, and synthetic approaches for these compounds, that possess all the properties required for success. Among the relevant considerations, poor yield, loss of solubility, unpredicted product formation, difficulties with selective iodination, stannylation, and/or radioiodination have all been factors. Moreover, the impact of the imaging moiety and its effect on cellular internalization of the probe where it can interact with normal tissue Hsp90 pools must also be considered. Unfortunately, even the best performers in cell-based studies will behave unpredictably in animal. Ultimately, we believe on the imaging side there is sufficient diversity of commercially available fluorophors of varying structural diversity to enable us to develop at least one T90i that will meet our criteria. Ideally, this molecule will carry both radioiodine and a fluorophore, the latter reporter enabling tumor cell selectivity within a biopsy to be determined by confocal microscopy.

6. Products.

On the basis of synthetic procedures described last year, several T90i, as well as their tin precursors, are available. Some of these also include a motif for optical imaging providing dual-modality imaging capability. A notable example of these compounds is HS113, described in detail in a previous section of this report.

7. Participants and Other Collaborating Organizations

Name	**Michael Zalutsky**
Project Role	**PI**
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Contribution to Project:	**Radiochemistry**

Project Role	**Xiao-Guang Zhao**
Researcher Identifier	**Not known**
Nearest person month worked:	**6**
Contribution to Project:	**Quality control, imaging and cell studies**

Collaborative Award:

Grants.gov ID Number	**W81XWH-15-1-0073**
Principal Investigator	**Timothy Haystead**
Performing Organization	**Duke University**
Contracting Organization	**Duke University**
Partner Budget Requested	**$549,500**
Direct Costs	**$350,000**
Indirect Costs	**$199,500**

My partnering PI, Dr. Tim Haystead (Duke University), submitted his own annual report.

8. **Special Reporting Requirements**.

None

COLLABORATIVE AWARDS:

9. **Appendices**

None