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

PRINCIPAL INVESTIGATOR:

Michael R. Zalutsky, Ph.D.

CONTRACTING ORGANIZATION:
Duke University
Durham, NC

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Michael R. Zalutsky, Ph.D.

E-Mail:zalut001@mc.duke.edu

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Duke University

2200 W. Main St., Suite 710 Box 104010 Durham 27705

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Hsp90 is a recognized oncogenic signal node whose up regulation/activation is associated with multiple forms of human cancer including 70% of all breast cancers. There are 17 Hsp9 inhibitors in clinical development for cancer, including SNX5422, developed by members of our team. In breast cancer, clinical studies have linked activation of Hsp90 and surface expression ectopic of Hsp90 (eHsp90) with poor outcomes in malignant breast tumors. We therefore hypothesized that selective targeting of eHsp90 on breast tumors presents an opportunity to not only discriminate indolent tumors from metastatic disease, but also offers a molecularly targeted radiotherapy approach for body wide tumor ablation with low normal tissue toxicity. To test this hypothesis, our laboratories developed a series of PET (Positron Emission Tomography) enabled tethered Hsp90inhibitors (Ti90s) that demonstrate exquisite selectivity in vivo for metastatic breast tumor cells. We also discovered that eHsp90 is rapidly internalized and can carry radionucleotide labeled Ti90 specifically into the breast cancer cell. In this proposal we will optimize conditions that enable two PET enabled Ti90s (HS-113 and HS-227) carrying radioiodine (^{124}I and ^{131}I) or ^{211}Astatine to be effectively delivered to malignant breast tumors using mouse models of triple negative, ER+ and HER2+ breast cancer. These studies will provide essential proof of concept data to move one or more PET enabled Ti90 into first in human clinical trials. Ultimately, we envisage a process in which a patient, after standard of care breast exam, is first evaluated for malignancy vs. indolent disease by PET imaging using ^{124}I-labeled Ti90. Then, in patients with malignancies detected in high contrast to normal tissues, targeted radiotherapy would be performed at patient-optimized doses of inhibitor labeled with the β-emitter ^{131}I or the α-emitter ^{211}At.

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1. **Introduction**.

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 (3). We also discovered that ectopically expressed Hsp90 is rapidly internalized and can carry these tethered inhibitors with specificity into 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 offer a molecularly targeted radiotherapy with a very favorable therapeutic index. We proposed to develop a series of tethered Hsp90 inhibitors capable of selectively delivering radiolabeled (^{124}I and ^{131}I) or ^{211}At to malignant tumor cells. We envisaged a process in which a patient facing either early stage or metastatic breast cancer is evaluated for Hsp90 overexpression on their tumor cells by positron emission tomography (PET) imaging of ^{124}I-labeled tethered inhibitors. Then, in patients with malignancies demonstrated to exhibit a sufficient level of ectopic Hsp90 expression, targeted radiotherapy would be performed at patient-optimized doses of inhibitor labeled with either the β-emitter ^{131}I or the α-emitter ^{211}At. This is an attractive strategy for breast cancer because the same molecule that detects ectopic Hsp90 can then be used for tumor selective radio ablation using our new compounds. In the same way Antibody Drug Conjugates can deliver chemotherapy selectively to HER2+ cells, our compounds would selectively deliver therapeutic doses of radiation to only malignant cells by manipulating the active uptake of ectopically expressed Hsp90 into cells.

2. **Key Words.**

Hsp90, Hsp70, PET, Pharmacokinetics, MTD, EGFR, ER, ^{131}I, ^{131}I , ^{211}At, targeted radiotherapy, nrIR, SNX5422, Hsp90 inhibitor, Breast Cancer, Ti90, eHsp90.

Overall Project Summary.

We hypothesized that ^{124}I-labeled tethered Hsp90 inhibitors (Ti90s) and PET imaging will allow non-invasive discrimination of malignant breast tumors from more benign disease. Furthermore, we hypothesized that this will also permit determination on an individualized basis, of the optimal dose of versions labeled with the therapeutic radionuclides ^{131}I or ^{211}At to achieve tumor ablation with minimal normal tissue toxicity. Our objectives were to test these hypotheses in proof of concept studies using various mouse models of human breast cancer. We anticipated that if successful, these studies will provide the basis for moving our approach into clinical trial.

3. **ACCOMPLISHMENTS**.

What were the major goals of the project?

Milestone Aim 1 **(50% complete).** Identification of a minimum of 3 cell selective lead molecules based on both the SNX2112 and PU-H71 scaffolds. We also anticipate that these leads will be derived from a larger chemical series that can also be further developed if unanticipated problems arise in Aims 2-4.

Milestone 1 Aim 2 (**75% complete**). Identification by PET and necropsy biodistribution tethered inhibitors with appropriate PK for use as PET imaging agents with ^{124}I and targeted radiotherapy with ^{131}I and ^{211}At.

Milestone 2 Aim 2 (**50% complete**). Demonstration that tethered [^{124}I]PU-H71 shows greater tumor selectivity compared with untethered [^{124}I]PU-H71 in multiple mouse models of metastatic breast cancer.

Milestone Aim 3. (**50% complete**) Demonstration of successful targeted radiotherapy with one or more tethered Hsp90 inhibitor carrying either ^{131}I or ^{211}At in in multiple mouse models of metastatic breast cancer.

What was accomplished under these goals?

A major goal of the funded research was to develop Ti90s that were amenable for duel imaging modalities of PET and optical, such that they could be readily interfaced with current mammography practice. To this end, with this funding, we extended our existing tethered Hsp90 synthetic platform to develop Ti90 molecules that could first be used for histology with the nonradioactive iodine-containing analogs, and then for noninvasive whole body PET imaging with the same molecules labeled with ^{124}I, and ultimately, for selective tumor

ablation with analogs labeled with ^{131}I. We dubbed this strategy the "see and cure" approach. As discussed in the original narrative, in mammography for example, a patient who requires a biopsy receives a micro dose of a Ti90 carrying non-radioactive iodine. If fluorescence microscopy of the biopsy slides reveals uptake of the probe, and this concurs with the pathology diagnosis, the patient would be offered the PET enabled, ^{124}I-labeled version for whole body imaging to identify evidence of disease progression. This could then be followed by targeted radiotherapy with the ^{131}I version. Note, that the physicochemical properties of the molecule are the same at all stages, just the isotope changes. This is greatly enabling for the clinical development pathway because the initial safety studies (GMP/GLP) prior to PET imaging can be carried out with nonradioactive material. With these goals in mind, the Haystead lab developed a synthetic pathway to derive a series of fluorescent probe structures also carrying iodine that could subsequently be converted into stannylated precursors for radioiodination. During the development of these probes, we discovered that placement of iodine within the ligand binding region inactive the molecule completely and 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 Ti90's we made, HS-113 and HS-227. The complete synthetic pathways of all our Ti90s containing an iodine (can be substituted with^{124}I for PET) 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 radioiodination of the tin precursors of HS-113 and HS-227 with ^{124}I. These methodologies will be disclosed in future submissions describing the utility of ^{124}I-labeled Ti90s for imaging tumors *in vivo*. Recent published work with non-radioactive fluorescent versions has enabled us to define the molecular mechanism by which PET imaging-amenable Ti90s recognize 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, more recent independent studies by the Ramanujam lab demonstrated direct uptake of a non-radioactive analog of HS-113, synthesized in the Haystead lab (HS-27), 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 seminal studies provide evidence that aggressive forms of breast cancer selectively express eHsp90 in humans. Collectively, these studies demonstrate that Ti90s 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+ve tumor cells by flow cytometry. In a recently completed DOD funded program, the Haystead lab successfully took a newly synthesized nrIR tethered Ti90 (HS-196) through the IND process (GMP manufacture, GLP animal toxicity and formulation). In 2017, the Lyerly group (Duke University) opened a 120 patient first in human solid tumor phase 1study to evaluate the safety and utility of HS-196 to detect malignancies *in vivo* (https://clinicaltrials.gov/ct2/show/NCT03333031). Probes like HS-196 will have no utility for whole body imaging or treatment. Light scattering of the emitted fluorescence, limits detection to tissue depths of ~0.5-1.0cm. However, lessons learned with HS-196 will be invaluable in guiding the future preclinical and clinical development of PET imaging-amenable versions of Ti90s. 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+ 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 masses 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 (<u>No DOD funds were used for these animal studies</u>) . In both examples, we observe discrete probe accumulation in secondary tumor masses consistent with prior published work 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 Ti90s from that of a radioiodinated form of the Hsp90 inhibitor PU-H71 reported by Larson and colleagues (49). 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

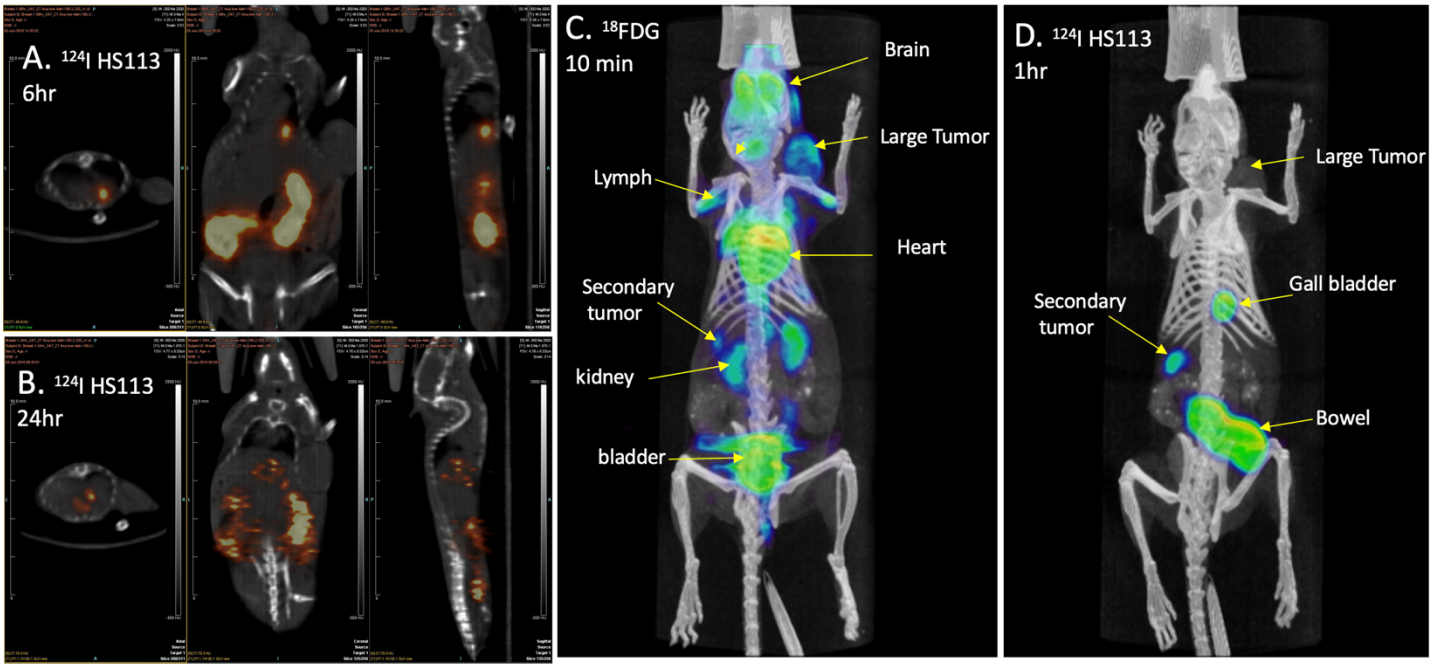

Figure 1. PET/CT images showing that ^{124}IHS-113 detects primary and secondary tumor masses in mouse models of HER2+ breast cancer. (**A**) and (**B**) shows a time course of a single mouse injected with a trace amount ^{124}IHS-113 and imaged by PET/CT at two time points. A mass is detected at 6 hours 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.

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.

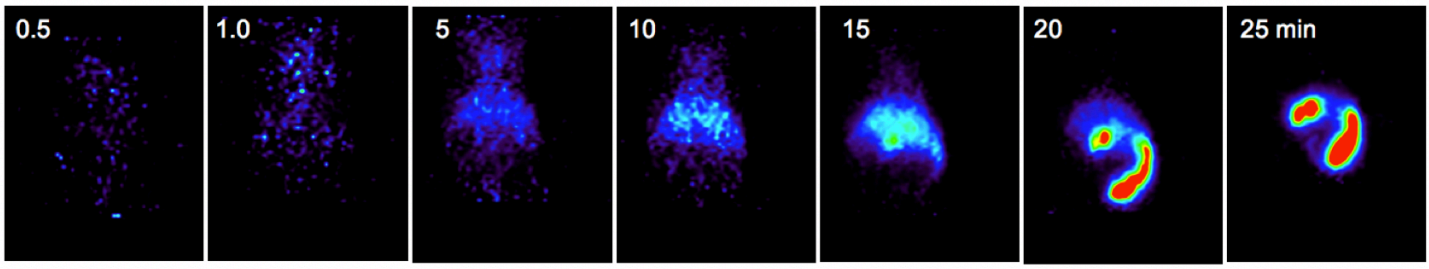

Figure 2. Time lapse sequence following the real time elimination of ^{124}IHS-113 by PET imaging in a mouse. The animal was injected with a tracer amount of ^{124}HS-113 and the signal followed continuously over 30 min. Images show the free probe rapidly collecting in the liver and condensing into the 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 (Fig. 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 Ti90 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 Ti90s persisted for some time in the circulation and were more slowly eliminated.

To date, we have synthesized 68 different Ti90s carrying various cargoes (see patents US9,738,643 B2, US 2015/0191471 A1, WO2014/025395 A). These include a full range of fluorophores of varying excitation and emission profiles. The full synthesis of these compounds is outlined in our patent filings as well as prior publications. With this funding, we identified at least two PET-amenable versions that we can reproducibly

synthesize, derivatize with a tin precursor for subsequent radiohalogenation, and the radiolabel. If either of these lead compounds fail, we are well placed to draw upon the large pool of backup molecules that may perform better *in vivo* in future work. Our work revealed some unexpected aspects of our PET-amenable Ti90s that could be advantageous in the development of both diagnostic and radiotherapeutic agents. With additional funding we will focus specifically on defining optimal delivery conditions that ensure both efficient tumor loading as well as rapid normal tissue clearance of unbound probe. We believe this is a critical goal in order to move the development of these probes forward to first in human phase 1 studies.

What opportunities for training and professional development has the project provided?

Nothing to Report.

How were the results disseminated to communities of interest?

Two patents were filed that have issued describing the full synthesis of all Ti90s synthesized in this project US9,738,643 B2, US 2015/0191471 A1, WO2014/025395 A.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report.

4. IMPACT.

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Nothing to Report.

6. PRODUCTS:

Two patents were filed that have issued describing the full synthesis of all Ti90s synthesized in this project US9,738,643 B2, US 2015/0191471 A1, WO2014/025395 A.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name	**Michael Zalutsky**
Project Role	**PI**
Researcher Identifier	**0000-0002-5456-0324**
Nearest person month worked:	**9**
Contribution to Project:	**Study design and analysis**
Project Role	**Ganesan Vaidyanathan**
Researcher Identifier	**0000-0003-3041-8275**
Nearest person month worked:	**12**
Contribution to Project:	**Radiochemistry**
Project Role	**Xiao-Guang Zhao**
Researcher Identifier	**Not known**
Nearest person month worked:	**22**
Contribution to Project:	**Quality control, imaging and cell studies**

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to Report

8. **SPECIAL REPORTING REQUIREMENTS**.

COLLABORATIVE AWARDS:

My partnering PI was Dr. Tim Haystead (Duke University) who will be submitting his own final report.

9. **APPENDICES**: