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TITLE: Quantitative PET Imaging with Novel HER3-Targeted Peptides Selected by Phage Display to Predict Androgen-Independent Prostate Cancer Progression

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# CONTRACTING ORGANIZATION: Massachusetts General Hospital Boston, MA 02114

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#### . SUPPLEMENTARY NOTES

#### 14. ABSTRACT

Funding from this award has permitted the development of a highly specific peptide that targets HER3 for prostate cancer imaging. The peptide was labeled with a PET imaging radionuclide and injected into mice bearing human prostate cancer. The peptide accumulated at high levels in the tumors, and excisional analysis revealed quantitative accumulation of the peptide in tumors that was linearly correlated with HER3 levels. Biodistribution analysis revealed low off-target accumulation and rapid clearance through the renal system, consistent with small peptides. The peptide represents a promising clinical lead for HER3 imaging in patients with castration resistant prostate cancer.

#### . SUBJECT TERMS

Castration Resistant Prostate Cancer, HER3, PET Imaging, Phage Display

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# 1. Introduction

The subject of this research is the design and testing of a PET imaging agent for the detection and characterization of castration resistant prostate cancer. It has recently been demonstrated that human epidermal growth factor-3 (HER3) is implicated in the transition from castration sensitive to castration resistant prostate cancer. As such, the purpose of this research is to select by phage display a suitable PET imaging agent for *in vivo* quantification and subsequently characterization of castration resistant prostate cancer. The selected peptide will be radiolabeled, injected into mice bearing prostate cancer and imaged.

# 2. Keywords

Castration Resistant Prostate Cancer, HER3, PET, Phage Display

### 3. Accomplishments

Major Goals (Research Specific Tasks)

Research-Specific Tasks:		
Specific Aim 1: Optimize and Characterize HER3 Targeted Peptide		
Subtask 1: Utilize phage display-mediated shotgun alanine scanning to isolate variants of the selected HER3 peptide that bind with higher affinity and specificity than the first- generation peptide.	1-9	Dr. Larimer
Subtask 2: Test three independent scaffolds, including PEG, amino acid and branched peptide constructs for improved HER3 targeting through a multivalent increase in peptide avidity.	6-12	Dr. Larimer
Subtask 3: Measure biodistribution and PET imaging uptake of the parent, affinity maturated and highest affinity scaffold in mice bearing HER3-expressing PC3 xenografts. Cell lines used: PC-3/ATCC (Internal Lab Stock)	6-15	Dr. Larimer
Milestone(s) Achieved: Identification of a peptide sequence and configuration that binds with <100 nM affinity and specific HER3 targeting in vivo	12-14	Dr. Larimer
Specific Aim 2: Determine the changes in radiolabeled HER3 peptide tumor uptake during androgen withdrawal therapy.	15-24	Dr. Larimer
Subtask 1: Perform PET imaging in mice bearing androgen sensitive resistant human prostate cancer xenografts throughout androgen withdrawal until tumor progression.	15-22	Dr. Larimer

Cell lines used: PC-3, MDA-PC-20/ATCC (Internal Lab Stock)		
Subtask 2: Correlate changes in peptide uptake with protein expression and cell signaling changes <i>ex vivo</i> . Cell lines used: PC-3, MDA-PC-2b (Internal Lab Stock)	18-24	Dr. Larimer
Milestone(s) Achieved: Positive prediction of castration resistant prostate cancer progression with supporting ex vivo	22-24	Dr. Larimer

*1) Major Activities*: The major activities included the development of a second-generation peptide by phage display that bound with higher affinity than the first-generation peptide. This peptide was tested both *in vitro* and *in vivo* and demonstrated to bind specifically and quantitatively to HER3.

2) Specific Objectives: The first specific objective was to develop a targeted peptide for HER3 than the original peptide. This was accomplished using a phage display selection in which 28 new peptide sequences were screened (Figure 1A). Interestingly, the selection converged upon a single HER3 specific sequence CLPTKFRSC. This sequence was shown to be highly specific

and conserved in HER3-avid phage, but not in non-specific phage isolated from the same selection. As such this peptide was pursued for further *in vitro* analysis.

The peptide was next synthesized using standard Fmoc chemistry conjugated to a biotin for affinity and specificity determination. The affinity was calculated at 271 nM, and the peptide had a greater than 10-fold specificity in comparison to other similar proteins such as EGFR and HER2 (Figure 2). Since the peptide was confirmed to bind to HER3, the next step was to confirm binding in the cellular context for which the imaging agent would be used. As such the peptide was tested with MDA-MB-453 HER3 positive cells and HCC-1954 HER3 negative cells. Cell binding was quantified ELISA and specific binding was demonstrated to HER3+ but not HER3 – cells with the HER3P1, which was not demonstrated using a control peptide (Figure 3A).



Furthermore, specific cellular binding was visualized using fluorescent microscopy, which confirmed specificity for HER3+ but not HER3- cells (Figure 3B). Given the excellent characteristics of the biotinylated peptide, the peptide was synthesized in the same manner, however NOTA was substituted for biotin in preparation for *in vivo* studies. The NOTA peptide was radiolabeled with <sup>68</sup>Ga and used in cell binding. The radiolabeled cell binding mimicked the

nuorescent ofnaing and confirmed that replacement of blotin with Ga-NOTA did not after peptide binding (Figure 3C).

The radiolabeled peptide was next prepared for in vivo PET imaging studies. The 22RV1 castration resistant prostate cancer cell line was chosen because it had been determined that it expressed high levels of HER3 protein suitable for PET imaging. Since all prostate cancers have HER3 expression, a breast cancer cell line was chosen as a negative control. PET imaging was performed, and very high uptake was visualized in the prostate cancer tumor, but not in the breast cancer tumor (Figure 4A). Furthermore, when the tumors were excised, protein analysis demonstrated high levels of HER3 in the prostate cancer and not in the breast cancer, consistent with PET imaging (Figure 4B-C). In fact, when the PET signal for each individual tumor was plotted against its corresponding HER3 protein level, the TBR correlated linearly with the amount of protein, indicating that the peptide was quantitatively imaging HER3 expression (Figure 4D). The peptide biodistribution was also analyzed, and the data indicated specific accumulation in tumors with minimal uptake in all normal tissues that were HER3 negative, and specific accumulation in HER3+ tissues. Additionally, the peptide was cleared renally, with relatively low levels of accumulation in comparison to other FDA-approved imaging peptides (Figure 5).

### 3) Significant Results or Key Outcomes

The first year of this award has resulted in significant progress towards a HER3 imaging peptide for prostate cancer. A new peptide with high affinity and sensitivity was developed. This peptide has been demonstrated to bind HER3, distinguish high HER3 from low HER3-expressing cells in vitro, and finally quantitatively detect levels of HER3 in vivo. These works have been presented at the annual SNMMI meeting, accepted for publication in





# Figure 5

а			b
	Organ	%ID/g	
	Tumor	0.50±0.18	gan F
	Blood	0.18±0.04	
	Heart	0.31±0.07	
	Lungs	0.70±0.14	rition conset estimes ritings
	Stomach	0.30±0.07	TUNO TUNOT'S TUNOTING TUNO
	Intestines	0.48±0.15	C 51
	Liver	0.70±0.23	BR 4
	Kidney	10.1±1.67	<sup>40</sup> P < 0.001
	Spleen	0.38±0.09	22 v
	Muscle	0.28±0.14	1 1 0
	Bone	0.67±0.38	1 2 3 4 PET TBR

Notecular imaging and Biology, and nave resulted in  $\cup$ 5 patent application being filed for the peptide technology developed in this grant.

# 4) Other achievements

Based on the data generated in this project, specific funding for clinical translation of this peptide was sought and approved through an internal funding mechanism at Massachusetts General Hospital. The peptide is being prepared now for IND application, followed by IRB application and finally imaging in 10 cancer patients. The anticipated timeline for this to occur is Late 2019 or early 2020.

# Major Goals (Training Specific Tasks)

*Attend Harvard Catalyst Courses*: I have taken and completed the Harvard Catalyst course Introduction to Translational Medicine, which was instrumental in helping to get the HER3 peptide described in this proposal funded for clinical translation.

Organize and deliver a research presentation between collaborating prostate research labs at *MGH*: A 2 hour seminar was organized between my mentor's (Dr. Mahmood) and Dr. Massimo Loda's lab at Dana Farber Cancer Institute to share research progress in November of 2016.

*Present Research at the SNMMI, AACR or PEGS National Meetings*: The work performed under this award was accepted for an oral presentation at the SNMMI annual meeting and I presented in June 2017.

# **Opportunities for Training and Personal Development**

This award has afforded me the opportunity to present and receive feedback on my research with established prostate cancer specific and imaging specific scientists in my field. I have accomplished this through presentations with my mentor (Dr. Mahmood) collaborators (Dr. Loda), attendees of the SNMMI meeting that I presented this work, and peer-reviewers of the publication that resulted from this award. This has greatly improved my scientific direction and made me a better researcher.

### Dissemination of Results to Communities of Interest

A portion of this work was presented at the SNMMI annual meeting during a time when patient advocates were invited to attend. This provided me with an opportunity to share my work with those who may be affected by my research.

### Plans to Accomplish Future Goals

In order to accomplish the remaining subtasks outlined in the statement of work, the following work will be performed

*Specific Aim 1- Subtask 2*: While PEGylation has been tested as described above, amino acid and branched peptide structures still need to be tested. These will be synthesized by Anaspec and tested in months 14-20.

*Specific Aim 2 – Subtask 1*: PC-3 and MDA-PC-2b cells will be implanted and imaged using the HER3P1 peptide that has been generated in Year 1 of this proposal.

*Specific Aim 2 – Subtask 2*: Following imaging of a portion of the mice from Subtask 1, the tumors will be excised and subjected to SDS-PAGE and Western blots for HER3 and other signaling proteins including the androgen receptor and MAPK and PI3K/AKT pathways investigated.

# 4. Impact

<u>Impact on the development of the principal disciplines of the project</u>. The peptide developed in this application is to the best of my knowledge the first HER3 imaging peptide. This represents a significant milestone in generating imaging agents that can be used to disseminate castration resistant prostate cancer at an early stage. Furthermore, this work represents the first HER3 imaging in prostate cancer and sets an important precedent for future work corroborating HER3 with castration resistant prostate cancer.

<u>Impact on Other Disciplines</u> The impact of this work on the field of molecular imaging is the demonstration that HER3 is a viable imaging biomarker in prostate cancer, and it may spur further research on imaging HER3 in prostate cancer by molecular imaging scientists.

<u>Impact on Technology Transfer</u> The technology developed by this grant has led to US patent application being filed for the technology. Furthermore, Massachusetts General Hospital has invested \$200,000 dollars in order to translate the peptide into humans in order to further investigate the efficacy of this peptide. This could in turn lead to a start-up being founded around the technology or licensing to a third-party for commercialization and wider dissemination of the product.

<u>Impact on Society Beyond Science and Technology</u> While in the end therapies are what are needed to cure prostate cancer, it is important to realize that characterization of these cancers is paramount to solving the problem. Cancer has been shown to a widely varied disease from patient to patient, and an increased public perception of the need for personalized medicine will help to improve care. I believe that by sharing this work as to one of the ways to characterize prostate cancer, I can help to spread the belief of investing in methods to personalize medicine and thus improve prostate cancer treatment.

### 5. Changes/Problems

<u>Changes in Approach and Reasons for Change:</u> Only slight deviations were made from the stated approach, and these did not change the fundamental approach to the project. One change was that a naïve peptide library was chosen for reselection based on characterization of the parent peptide. This resulted in a much-improved peptide sequence that was free of non-specific binding. The second minor change was to use 22RV1 cells instead of PC-3 cells, as the 22RV1 cells had much higher levels of HER3 than PC3.

<u>Actual or anticipated problems or delays and plans to resolve them</u>: Our PET/CT scanner was not functioning from March through July of 2017, however an alternative PET/MR has been found that will permit continuation of the project. No future problems are anticipated.

Significant changes in the use or care of human subjects, vertebrate animals, biohazards and/or select agents: Nothing to report.

### 6. Products

Journal Publications:

Larimer BM, Phelan N, Wehrenberg-Klee E, Mahmood U. Phage Display Selection, *In vitro* Characterization and Correlative PET Imaging of a Novel HER3 Peptide. Molecular Imaging Biology 77.9 (2017): 2318-2327. PubMed PMID: 28733706

Books or other non-periodical, one-time publications Nothing to report.

Other publications, conference papers, and presentations: International Conference Presentation: Lammer, Benjamin, et al. Fnage display selection of a novel HEKS FE1 imaging peptide for targeted therapy resistance prediction." *Journal of Nuclear Medicine* 58. supplement 1 (2017): 690-690.

Websites or Other Internet Sites: Nothing to report.

Technologies: Nothing to report.

Inventions, patent applications, and/or licenses: Novel HER3 Peptide for Imaging and Radiotherapy; US Patent Pending 62/440,052

Other Products: Nothing to report.

### 7. Participants and Other Collaborating Organizations

Individuals Working on the Project Name: Benjamin Larimer Project Role: PI Researcher Identifier: 0000-0002-1288-7206 Nearest Person Month Worked: 7 Contribution to Project: Dr. Larimer conceived the project, performed all experiments, wrote the manuscript. Name Umar Mahmood Project Role: Lead Mentor Research Identifier: NA Nearest Person Month Worked: 0 Contribution to Project: Dr. Mahmood provided mentorship to Dr. Larimer and assisted in planning experiments and writing of the manuscript.

<u>Changes in the Active Other Support of The PD/PI</u> Dr. Larimer is now an investigator on an NIH grant to Dr. Mahmood (1R01CA214744) for 1.2 calendar months.

### 8. Special Reporting Requirements

Nothing to report.

### 9. Appendices

Attached appendices include a copy of the manuscript, abstract, and patent application referenced in this report.



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# **RESEARCH ARTICLE**

# Phage Display Selection, *In Vitro* Characterization, and Correlative PET Imaging of a Novel HER3 Peptide

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#### Abstract

Purpose: HER3 (ERBB3) is a receptor tyrosine kinase that is implicated in treatment resistance across multiple cancers, including those of the breast, lung, and prostate. Overexpression of HER3 following targeted therapy can occur rapidly and heterogeneously both within a single lesion and across sites of metastasis, making protein quantification by biopsy highly challenging. A global, noninvasive methodology such as positron emission tomography (PET) imaging can permit serial quantification of HER3, providing a useful approach to monitor HER3 expression across the entire tumor burden both prior to and following treatment. PET imaging of HER3 expression may permit a more personalized approach to targeted therapy by allowing for detection of HER3-mediated resistance, in addition to informing clinical trial patient selection for novel therapies targeting HER3. Procedures: Phage display selection targeting the HER3 extracellular domain was performed in order to develop a peptide with optimal blood clearance and highly accurate HER3 quantification. Results: The selection converged to a consensus peptide sequence that was subsequently found to bind HER3 with an affinity of 270 ± 151 nM. The peptide, termed HER3P1, was bound with high selectivity to HER3 over other similar receptor tyrosine kinases such as EGFR and HER2. Furthermore, HER3P1 was able to distinguish between high and low HER3-expressing cells in vitro. The peptide was radiolabeled with Ga-68 and demonstrated to specifically bind HER3 by in vivo PET imaging. Uptake of [68Ga]HER3P1 was highly specific for HER3-positive tumors, with tumor-tobackground ratios ranging from 1.59-3.32, compared to those of HER3-negative tumors, ranging from 0.84–0.93. The uptake of  $[^{68}$ Ga]HER3P1 also demonstrated high (P < 0.001) correlation with protein expression as quantified by Western blot and confirmed by biodistribution.

*Conclusions:* HER3P1 accurately quantifies expression of HER3 by PET imaging and has potential utility as a clinical imaging agent.

Key words: HER3, PET, Phage display, Peptide

### Introduction

Molecularly targeted cancer therapy, while providing benefits for a number of indications, has failed to produce durable responses in a majority of patients [1, 2]. While no single mechanism is entirely responsible for targeted therapy resistance, a recurring theme is the upregulation of feedback loops that circumvent blockade of oncogenic signaling pathways [3, 4]. Feedback loop signaling can be accomplished through alternative activation of a homologous pathway or through overexpression of a secondary oncogenic protein [5]. One such protein, human epidermal growth factor receptor 3 (HER3), has been implicated in

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targeted therapy resistance in a number of malignancies including breast, lung, and prostate cancer [4].

HER3 is a member of the epidermal growth factor (EGF) family of receptors that includes EGFR. HER2/ErbB2/neu. HER3/ErbB3, and HER4/ErbB4 [6]. The EGFR family canonically functions through ligand binding followed by dimerization, phosphorylation, and downstream signaling through the MAPK and PI3K/AKT pathways [6-8]. These receptors are major drivers of tumorigenesis, and multiple targeted therapies that block EGFR and HER2 are approved by the Food and Drug Administration [1, 9, 10]. Often, however, therapies that target EGFR and HER2 are only transiently effective, which may be due, in part, to a subsequent increase in membrane HER3 expression [4]. Upregulation of HER3 expression and transphosphorylation may lead to escape from therapeutic inhibition through the PI3K/AKT pathway, rendering initial targeted therapy ineffective. Although HER3 has been less explored historically due to its mutated kinase domain, concurrent discoveries that its intracellular domain can in fact signal through transphosphorylation and that it is overexpressed in patients with resistance to a number of targeted therapies have brought it to the forefront of targeted therapy development [4, 11, 12]. In fact HER3-mediated therapeutic resistance may not be limited to therapies that target EGFR or HER2, as evidence has also emerged to link HER3 with castration-resistant prostate cancer [13, 14].

Given its central role in targeted therapy resistance, a method to detect HER3 expression could provide oncologists with critical data necessary to inform treatment. While biopsy has traditionally been used to characterize protein expression, the rapid and transient nature of HER3 expression would require serial biopsies, which are not routinely performed, in order to accurately detect changes in expression [15]. A more tractable option to detect and quantify HER3 would be through positron emission tomography (PET) imaging. PET imaging provides rapid, non-invasive, and quantitative measurement of protein expression and can be performed at multiple time points in order to assay pretreatment and post-treatment levels of HER3 [16]. Due to the importance of HER3 in cancer therapeutic resistance biology, a clinically translatable HER3 PET imaging agent could help to diagnose targeted therapy resistance prior to tumor progression. This information could also be used to better select patient populations for current HER3 therapy clinical trials, which have yet to be successful despite growing evidence of the role of HER3 in tumor progression.

In order to generate a suitable clinically translatable imaging agent, the molecule should not only have high affinity and specificity for its target, but also demonstrate rapid clearance from the bloodstream and excellent tumor penetration. Peptides, which have a molecular weight of approximately 1–5 kDa and are non-immunogenic, demonstrate low off-target accumulation and are cleared through the renal system [17]. Given these favorable properties, small peptides or peptidomimetics such as DOTATOC/

TATE and PSMA are used clinically or in clinical research in both the USA and Europe [18, 19]. In order to develop a peptide that targets HER3, a combinatorial selection technique termed bacteriophage (phage) display was used [20]. Phage display utilizes libraries of up to one billion unique peptides encoded in the DNA of filamentous viruses to pan for high affinity and specific peptides and has been used to successfully identify peptides that target HER2, the  $\alpha_V\beta_3$ integrin, and numerous other targets [21–23].

It was hypothesized that phage display would permit selection of a HER3 peptide that would have favorable properties for *in vivo* PET imaging. Therefore, we performed a phage display selection against the extracellular domain of HER3, and a peptide sequence that targeted HER3 was discovered. The peptide was characterized *in vitro* prior to analysis in HER3-expressing cancer models *in vivo*. The HER3 peptide was able to discriminate with high accuracy not only between high and low HER3 tumors, but also individual levels of HER3 expression among high-HER3 tumors. Because it demonstrates exquisite sensitivity in HER3 quantification, the novel HER3 peptide represents an attractive clinically translatable imaging agent for noninvasive detection of HER3 levels.

### Materials and Methods

#### Materials

All chemicals and solvents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. MDA-MB-453, HCC1954, and 22Rv1 cells were purchased from American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 media supplemented with 10 % fetal bovine serum and 1 % penicillin and streptomycin.

#### Phage Display Selection

A cysteine-constrained randomized 7-mer library (New England Biolabs, Ipswich, MA) was utilized for the phage display selection. HER3 extracellular domain (ECD) (R&D Systems, Minneapolis, MN) was conjugated with long chain biotin (Thermo Scientific, Waltham, MA) utilizing standard NHS ester chemistry and purified by size exclusion chromatography with a 10-kDa molecular weight cutoff column (Genesee Scientific, San Diego, CA). Purified, biotinylated HER3 was bound to Dynabeads M-280 streptavidin beads (Thermo Scientific) and blocked with 1 % non-fat dry milk in Tris-buffered saline (TBS) plus 0.1 % Tween 20 (TBST). An aliquot of  $2 \times 10^{11}$  plaqueforming units of phage was added to the beads and incubated for 1 h at 37 °C. The beads were then washed 10 times with TBST and eluted with 0.2 M glycine, pH 2.2, and subsequently neutralized by addition of 1 M Tris pH 8 to pH 7.2 The output was then amplified in ER2738 Escherichia coli (New England Biolabs) for 4.5 h at 37 °C

followed and purified by the standard polyethylene glycol (PEG)/NaCl method [24]. The selection was performed for three rounds and following the third round, individual phage plaques were picked for analysis by enzyme-linked immunosorbant assay (ELISA).

#### Individual Phage Characterization

Individually selected phages were amplified for 4.5 h at 37 °C and supernatant was collected following centrifugation. HER3 ECD and bovine serum albumin as a control were adsorbed to Nunc Maxisorp 96-well plates (Sigma, St. Louis, MO). Supernatants corresponding to individual phages were added to HER3 and bovine serum albumin (BSA) wells and allowed to bind for 1 h at 37 °C. After incubation with peptide, wells were washed six times with TBST and bound phages were detected by the addition of a horseradish peroxidase (HRP)-conjugated anti-M13 antibody (GE Health Sciences). After a subsequent washing, 2.2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) substrate was added to wells and absorbance at 405 nm quantified by a Promega Glomax spectrophotometer (Promega, Madison, WI). The relative binding was compared to a control phage bearing no peptide and represented by the heat map in Fig. 1a. The phages with the five best binding ratios of HER3 to BSA were chosen for amplification and purification to ensure no other supernatant factor was obscuring the signal. Following purification of the phages, ELISA was performed in the exact same manner and the absorbance of each phage to either HER3 or BSA was quantified by the spectrophotometer. Phagemid DNA from each of the five phages in addition to three phages with no binding per the initial ELISA was recovered by plasmid miniprep isolation and automated Sanger DNA sequencing was performed by the CCIB DNA Core Facility at the Massachusetts General Hospital (Cambridge, MA).

#### *In Vitro HER3 Affinity and Specificity Characterization*

After determination of a consensus peptide-binding sequence, the peptide sequence was covalently linked to a biotin-conjugated N-terminal triglycine linker using standard Fmoc chemistry. The purity and molecular weight were determined by high-performance liquid chromatography and mass spectrometry. In order to confirm HER3 affinity, the peptide was analyzed for binding to HER3 ECD immobilized to Nunc Maxisorp 96-well microtiter plates. Following blocking with 1 % non-fat dry milk, increasing concentrations of peptide were incubated with target protein for 1 h at 37 °C. Wells were washed with 0.1 % TBST, and bound peptide detected by addition of HRP-conjugated streptavidin (Abcam, Cambridge, UK), washing and ABTS substrate incubation for 10 min. Absorbance at 405 nm was read by the spectrophotometer. In order to compare the specificity for HER3 against similar family members EGFR and HER2, extracellular domains of both EGFR and HER2 were immobilized and subjected to the same peptide ELISA at a single peptide concentration of 250 nM. Plates were washed and bound peptide once again detected by addition of streptavidin-HRP and ABTS and absorbance read at 405 nm.

#### HER3 Peptide Cellular Binding and Specificity Analysis

Although the peptide bound pure HER3, its binding capability in the context of the extracellular microenvironment needed to be analyzed prior to *in vivo* analysis. Two cell lines, MDA-MB-453 breast cancer cells which express moderate levels of HER3 and HCC-1954 cells which express low levels of HER3, were chosen to assess the ability of the peptide to discriminate between relatively close expression levels *in vitro*. Cells were seeded at a density of  $1 \times 10^5$  cells/well in 96-well plates (Fisher) and grown for 24 h in fetal bovine serum supplemented medium. HER3 peptide or a control peptide was added at 250 nM in media and incubated for 1 h at 37 °C. Cells were washed 3× with PBS and bound peptide detected by addition of streptavidin-HRP. Cells were once again washed and bound peptide detected by addition of ABTS and absorbance measured at 405 nm.

Peptide binding was confirmed visually by fluorescent microscopy. MDA-MB-453 and HCC-1954 cells were fixed in 10 % formalin and dried onto microscope slides overnight. Following rehydration with TBS, slides were blocked with 2 % BSA in TBS for 1 h prior to addition of peptide at a concentration of 250 nM in 0.1 % TBST and incubation at room temperature for 1 h. Cells were washed 3× with 0.1 % TBST and neutravidin-AlexaFluor488 was added to cells and incubated at room temperature for 1 h. Cells were washed and mounted with 4',6'-diamidino-2-phenylindole, dihydrochloride (DAPI)-containing Vectashield mounting media (Vector Labs, Burlingame, CA). Peptide binding was visualized by inverted microscope (Olympus, Tokyo, Japan).

In order to analyze whether the substitution of biotin for 1,4,7-triazacyclononane-N,N',N"-triacetic acid (NOTA), a bifunctional chelator that would permit radiolabeling and in vivo assessment of the peptide by PET imaging, would affect HER3P1 binding, the NOTAconjugated HER3 peptide was synthesized. Synthesis occurred in the same manner as the biotinylated peptide, with NHS-ester NOTA (Macrocyclics, Dallas, TX) being substituted for biotin. The peptide was radiolabeled with Ga-68 eluted from a Ge-68/Ga-68 generator (Radiomedix, Houston, TX) in 0.05 M HCl. The pH of the eluate was adjusted to approximately 4 by 2 M 2-[4-(2-hydroxyethyl)piperazin-1yl]ethanesulfonic acid buffer pH 8 and 100 ng of peptide reacted with the elution for 10 min at room temperature. Peptide was purified from free Ga-68 by reverse-phase C18 cartridge (Waters, Milford, MA) and purity determined by ITLC. Ten



**Fig. 1. a** Phage display selection for HER3 peptide heat map of standardized phage supernatant binding to HER3 and BSA as control. *Green* indicates high binding and *red* indicates low binding. **b** Normalized binding of the five highest phages from supernatant phage ELISA; *bars* represent the mean of six replicates  $\pm$  SEM. **c** Sequences of phages with high binding (1, 5, 17, 19, 26) and low binding (7, 22, 27) to HER3. \*\*P < 0.01.

microcuries of purified [ $^{68}\text{Ga}$ ]HER3P1 was then added to 1  $\times$  10 $^5$  MDA-MB-453 cells and incubated for 1 h. After incubation, cells were washed 3× with TBS and bound radioactivity quantified by a Wallac gamma counter (Perkin Elmer, Waltham, MA). As a control, excess (100  $\mu\text{M}$ ) peptide was added to cells and bound peptide measured in the same manner.

#### Positron Emission Tomography Imaging and Ex Vivo Correlation

The [<sup>68</sup>Ga]HER3P1 was labeled and purified in the same manner as described for in vitro analysis, and the final preparation was diluted in normal saline to approximately 300 MBq for injection into mice. Nu/nu mice bearing either 22RV1 (high HER3-expressing prostate cancer) or HCC-1954 (low HER3-expressing breast cancer) tumors were implanted in the right upper flank of mice and grown to 5-7 mm. Radiolabeled HER3 peptide was injected intravenously and allowed to circulate for 1 h prior to PET imaging. PET images were acquired on a Triumph microPET/CT (Trifoil, Chatsworth, CA) for 15 min in list mode, followed by CT acquisition. Images were constructed using 3D-MLEM (20 subsets) and corrected for scatter and randoms. The tumor and blood uptake was calculated in a 3D region of interest drawn around the tumor and heart, respectively, using CT guidance. Images were post-processed using VivoQuant (InviCRO, Boston, MA).

Following PET acquisition, tumors and relevant organs were removed from mice and weighed, and the total activity for each was quantified by a Wallac gamma counter (Perkin Elmer, Waltham, MA). After radioactive decay, tumors were lysed and analyzed by Western blot for correlation of tumor uptake to HER3 expression normalized to  $\beta$ -actin. HER3 (sc-81455, Santa Cruz Biotech, Dallas, TX) and  $\beta$ -actin (13E5, Cell Signaling, Danvers, MA) antibodies were used to detect protein followed by an HRP-conjugated goat-antirabbit secondary antibody (Abcam) and detection by SignalFire chemiluminescent substrate (Cell Signaling).

#### Statistical Analysis

For comparison of phage clones against BSA and peptide specificity against other receptor tyrosine kinases, a one-way ANOVA with a Dunnett test to correct for multiple comparisons was performed. A sigmoidal dose-response curve was fit to the peptide affinity data using GraphPad Prism 6.0. For peptide cell binding, a two-way ANOVA with Sidak's multiple comparisons test was performed. A linear regression was fit to HER3 TBR *versus* Western blot data, with correlation significance calculated by a Pearson correlation. An unpaired *t* test was used to compare HER3 expression between 22RV1 and HCC-1954 tumors.

### Results

#### Phage Display Selection

Following three rounds of selection, 28 individual phage clones were selected for initial ELISA screening. Phage binding was measured as absorbance and normalized to control phage. Of the resulting 28 phages screened, 23 had greater binding to HER3 than the control phage, and the five phages with the largest HER3 versus BSA differentials were chosen for further analysis (Fig. 1a). Each of these five phages had significantly higher background subtracted binding to HER3 as quantified by absorbance (range = 0.177-0.253) than the control phage, which had an absorbance of  $0.007 \pm 0.002$  (P < 0.001) (Fig. 1b). Given the significant specificity of each phage, all five were sequenced to ascertain the amino acid composition of their displayed peptide. Surprisingly, each of the sequenced phage displayed an identical peptide. To further confirm that the convergence of the selection was not resultant from a target-unrelated peptide, three phages which did not demonstrate HER3-specific binding were also sequenced, and each had a different peptide sequence than the convergent sequence (Fig. 1c).

#### In Vitro Peptide Analysis

Since each of the top five phage clones analyzed displayed an identical sequence, this sequence was chosen for in vitro analysis to confirm peptide binding outside of the phage scaffold. For initial characterization, the peptide (HER3P1) was conjugated to biotin through a triglycine spacer. The biotinylated-HER3P1 peptide was first analyzed with purified HER3 ECD to obtain an approximate binding affinity. HER3P1 bound in a sigmoidal manner, with an affinity of  $270 \pm 151$  nM (Fig. 2a). In order to ensure that the peptide was specific for HER3 and not EGFR or HER2, similar receptor tyrosine kinases also expressed on the cellular surface of many tumors; the binding of HER3P1 was tested against each receptor. The absorbance of HER3P1 binding to HER3 was  $0.82 \pm 0.03$ , whereas binding to EGFR was  $0.21 \pm 0.09$ , HER2 was  $0.22 \pm 0.07$ , and BSA was  $0.15 \pm 0.01$  (*P* < 0.001 for all), indicating that the peptide was highly specific for HER3 and suitable for cell binding analysis (Fig. 2b).

Although the peptide bound HER3 in isolation, it was critical to assess its ability to interact with the receptor in a cellular context. To assay cellular binding, biotinylated-HER3P1 or a non-specific peptide was incubated with either MDA-MB-453 human breast cancer cells, which express moderate levels of HER3, or HCC-1954 cells which have lower levels of HER3 expression. Cell binding for HER3P1 was quantified and absorbance for MDA-MB-453 was  $0.29 \pm 0.06$ , whereas it was significantly lower for HCC-1954 cells 0.13  $\pm$  0.06 (P < 0.05). Additionally, HER3P1 binding was significantly higher to MDA-MB-453 cells than the control peptide (0.12  $\pm$  0.06, P < 0.05), whereas there was no difference between the control peptide and the HER3P1 for binding to HCC-1954 cells (Fig. 3a). The strong selectivity of the peptide was also apparent using fluorescent microscopy, with high binding to MDA-MB-453 cells and almost no visualization of HCC-1954 cells (Fig. 3b). Having demonstrated the HER3-targeting capabilities of the peptide sequence, confirmation of peptide binding after substitution of NOTA and radiolabeling was sought by competitive cell binding. The [<sup>68</sup>Ga]HER3P1 bound to MDA-MB-453 cells, binding was significantly (P < 0.05) blocked by the addition of excess unlabeled NOTA-HER3P1, indicating specific peptide binding (Fig. 3c).

#### Murine PET Imaging and Ex Vivo Analysis

After characterizing the ability of the HER3P1 to accurately distinguish between HER3 expression *in vitro*, the peptide was synthesized and conjugated to NOTA for *in vivo* analysis. NOTA-HER3P1 peptide was radiolabeled with Ga-68 and purified to greater than 95 % purity by reverse phase chromatography, as determined by ITLC. The specific activity of the labeled peptide was 296  $\pm$  25.9 MBq/mg. A human prostate castration-resistant cancer cell line 22RV1, was chosen because of its high levels of HER3 expression, and HCC-1954 cells remained as the negative control for PET imaging studies. PET imaging demonstrated high [<sup>68</sup>Ga]NOTA-HER3P1 tumor uptake in 22RV1 tumor-bearing mice, with significantly lower levels in HCC-1954 tumor-bearing mice (Fig. 4a). Off-target peptide accumulation was minimal, with uptake in the kidneys and bladder



Fig. 2. Biotin-HER3P1 characterization against purified target. **a** Peptide ELISA with increasing concentrations of HER3P1 demonstrates saturable binding and an affinity of  $270 \pm 0.151$  nM. *Triangles* represent the mean of six replicates  $\pm$  SEM. **b** Measured HER3-1 absorbance against two similar receptor tyrosine kinases and BSA, demonstrating approximately tenfold selectivity over closely related proteins. *Bars* represent the mean of six replicates  $\pm$  SEM. \*\**P* < 0.01.



**Fig. 3.** Biotin-HER3-1 cell binding. **a** Quantitative measurement of HER3-1 binding to moderate (MDA-MB-453) and low (HCC-1954) HER3-expressing cells reveals significant differentiation by HER3-1 between cell types, in addition to significantly higher binding to a moderate HER3 cell line than a control peptide. **b** Fluorescent microscopy visualizes peptide binding to MDA-MB-453 cells, with little binding to HCC-1954 cells. **b** Competitive cell binding analysis of [<sup>68</sup>Ga]HER3P1 demonstrates specific binding to MDA-MB-453 cells; *bars* represent the mean of four replicates with *error bars* representing SEM. \**P* < 0.05.

consistent with the normal route of peptide clearance. Standardized tumor-to-blood ratios (TBR) for high HER3 22RV1 ranged from 1.60 to 3.32 (n = 4), whereas low HER3 HCC-1954 tumors ranged from 0.69 to 0.94 (n = 4).

Following PET acquisition, tumors were excised and HER3 and  $\beta$ -actin were quantified by Western blot (Fig. 4b). The HER3: $\beta$ -actin ratio for each tumor was plotted against

its corresponding TBR and fit to a linear regression with a goodness of fit  $R^2 = 0.96$  and a Pearson correlation *P* value of less than 0.0001. This data confirmed that the HER3P1 uptake was highly correlated with HER3 expression and accurately quantified total HER3 *in vivo* (Fig. 4c). Additionally, biodistribution analysis of HER3P1 was performed. Sites of accumulation included the HER3+ tumors



Fig. 4. [<sup>68</sup>Ga]NOTA-HER3–1 PET imaging and tumor HER3 expression correlation. **a** Sagittal and axial maximum intensity projections of the radiolabeled peptide in high HER3-expressing 22RV1 and low HER3-expressing HCC-1954 tumors. Peptide uptake is visualized in the tumor and kidneys, which are the main route of clearance. **b** Western blot analysis of HER3 and  $\beta$ -actin for tumors excised from mice following PET imaging. **c** Quantification of HER3: $\beta$ -actin ratio for 22RV1 and HCC-1954 tumors; *bars* represent the mean of four replicates ± SEM. **d** Linear regression with 95 % confidence intervals for each tumor comparing PET TBR to *ex vivo* HER3: $\beta$ -actin ratio. Linear significance was determined by Pearson's correlation. \*\*P < 0.01; \*\*\*P < 0.0001.

 $(0.50 \pm 0.18 \% \text{ ID/g})$  and HER3+ organs such as the stomach  $(0.30 \pm 0.07 \% \text{ ID/g})$ , intestines  $(0.48 \pm 0.15 \% \text{ ID/g})$ , and lungs  $(0.70 \pm 0.14 \% \text{ ID/g})$ , in addition to kidneys  $(10.1 \pm 1.67 \% \text{ ID/g})$  as a route of clearance (Table 1). Accumulation in off-target organs was not due to blood accumulation, as tumor-to-organ ratios were significantly lower than tumor-to-blood ratios (P < 0.05 stomach, P < 0.001 intestines and lungs, Fig. 5a). Comparison between PET TBR and biodistribution TBR was highly correlated, as determined using Pearson correlation (P < 0.001, Fig. 5b).

### Discussion

The role of HER3 in targeted therapy resistance has been well documented, and efforts to pharmacologically inhibit its activity are currently being explored. Clinical trials examining the efficacy of anti-HER3 antibodies including patritumab (NCT02134015), MM-121 (NCT00734305), U3-1402 (NCT02980341), and GSK2849330 (NCT01966445) are ongoing or have been completed recently, with limited success to date [25]. One limiting factor for the administration of such agents is identifying patient populations most likely to benefit from the therapy. HER3 is particularly challenging to quantify by biopsy, because of its highly heterogeneous temporal and spatial expression. As drug approval continues to trend toward including a companion diagnostic with novel targeted therapies, an accurate method to quantify expression of HER3 to guide therapy is of paramount importance. PET imaging, which provides a global and repeatable methodology to assess target expression, is highly compatible with HER3 expression diagnosis. In order to facilitate such an assay, a novel HER3 peptide was selected by phage display and characterized for quantitative PET imaging of HER3-expressing tumors in murine models of multiple cancers.

Phage display, which is routinely used to screen for novel peptide ligands of various targets, was undertaken to identify a suitable peptide for HER3 imaging. Traditionally, phage display involves multiple rounds of selection, followed by screening of individual ligands to determine the highest affinity and specificity peptide among a host of candidates

Table 1. Biodistribution in all relevant organs depicted as the mean injected dose per gram of tissue (%ID/g) of four 22RV1 tumor-bearing mice  $\pm$  SD

Organ	% ID/g tissue ± SD		
Tumor	$0.5 \pm 0.18$		
Blood	$0.18\pm0.04$		
Heart	$0.31 \pm 0.07$		
Lung	$0.70\pm0.14$		
Stomach	$0.30\pm0.07$		
Intestines	$0.48 \pm 0.15$		
Liver	$0.70 \pm 0.23$		
Kidney	$10.1 \pm 1.67$		
Spleen	$0.38\pm0.09$		
Muscle	$0.28 \pm 0.14$		
Bone	$0.67 \pm 0.38$		

[26]. This process of selection and screening was undertaken for HER3, but all suitable peptides had identical sequences. Although this is not unique, it is relatively rare that a selection would converge on a single peptide sequence. Given the prevalence of phage display selections that are rendered unusable by contamination with target-unrelated peptides, sequences of non-binding phages were also obtained in order to determine if a single phage with a potential selective growth advantage dominated the selection [27]. However, the non-HER3 binding phages sequenced all had sequences different from both the HER3-avid phage and each other, limiting the possibility of a growth-advantaged phage dominating the selection. Further in vitro characterization of the displayed peptide conjugated to biotin demonstrated high affinity binding of 270 nM to HER3, with greater than tenfold specificity compared to that of



**Fig. 5.** Biodistribution and correlation of [<sup>68</sup>Ga]NOTA-HER3-1. **a** Tumor-to-organ ratios calculated by biodistribution values from results in **a** demonstrating specific accumulation in murine sites of non-tumor tissue HER3 expression. **b** Linear regression of TBR from both biodistribution *versus* PET imaging. Significance of linearity determined by Pearson's correlation. \*P < 0.05; \*\*\*P < 0.001.

other proteins, including the other similar receptor tyrosine kinases EGFR and HER2. Combined with specific binding of the peptide to HER3-expressing cancer cells, the peptide warranted further *in vivo* analysis.

Two tumor cell lines, 22RV1, a castration-resistant prostate cancer cell line with high levels of HER3, and HCC-1954, a breast cancer cell line with low levels of HER3, were chosen as models for binding analysis. PET imaging revealed high tumor-to-blood ratios in the HER3positive 22RV1 cell line, with background levels in HER3negative HCC-1954 tumors. Furthermore, *ex vivo* analysis of the tumors used for PET imaging provided a highly significant correlation between protein expression and PET TBR, indicating an accurate and robust method of HER3 quantification suitable for exploration in both pre-clinical and clinical trials.

To date, a small number of HER3 targeted PET imaging agents have been explored, with two reaching clinical trial. One clinical study has been performed by Lockhart et al. utilizing <sup>64</sup>Cu-labeled DOTA-conjugated patritumab to determine dosimetry and receptor occupancy; however, no correlation between tumor uptake and *ex vivo* immunohistochemical analysis could be determined, and specific accumulation measured by tumor-toblood ratio was approximately 1 [28]. One trial (NCT02345174) has utilized PET imaging of an <sup>89</sup>Zr-labeled therapeutic antibody, GSK2849330, prior to administration of the same unlabeled antibody, but no results have been posted yet. Thus, there remains a need for an accurate PET imaging agent to quantify HER3 expression, which may help expedite the clinical approval of current or novel HER3 therapies.

Pre-clinically, a number of antibodies, antibody fragments, and affibodies have been used to image HER3 with a variety of PET isotopes including F-18, Ga-68, Cu-64, and Zr-89. Although antibodies provide accurate quantification, slow clearance prevents serial imaging and may limit clinical application [29]. The HER3 affibody ZHER3:8698 is cleared rapidly and may represent a suitable HER3 clinical imaging agent, but high renal uptake limits repeat imaging [30]. One small molecule pan-RTK inhibitor has been radiolabeled for imaging; however, the cross-reactivity with other RTKs greatly reduces its specific HER3 utility [31]. Given the current status of HER3 imaging agents, a peptidebased imaging agent with high specificity and minimal background uptake represents a strong candidate for clinical translation.

Radiolabeled peptides, and specifically Ga-68 labeled peptides, have a strong presence in clinical targeted imaging. [<sup>68</sup>Ga]DOTATOC and DOTATATE are both in use clinically, with the latter gaining FDA approval in 2016. The rapid pharmacokinetics and high tumor-to-background ratios of peptides have facilitated highly accurate quantitation of target expression, permitting not only detection but also characterization for therapeutic optimization of drugs such as Lutathera and Octreotide. The fundamental imaging paradigm surrounding these peptides provides a framework for future clinical translation of peptides.

### Conclusion

HER3P1 represents a highly accurate peptide-imaging agent for HER3 with low off-target accumulation favorable for PET quantification. HER3 represents a critical protein in targeted therapies for a number of cancers, and its highly dynamic expression requires an equally dynamic approach to quantification. HER3 imaging with HER3P1 may represent an ideal method to quantify HER3 and provide critical cellular feedback analysis. As such, HER3P1 represents a novel HER3 imaging peptide with considerable promise as a clinical imaging agent.

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#### **Compliance with Ethical Standards**

#### Conflict of Interest

The authors declare that they have no conflict of interest.

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# Phage display selection of a novel HER3 PET imaging peptide for targeted therapy resistance prediction.

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### Abstract

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**Objectives:** The receptor tyrosine kinase HER3 (ERBB3) is upregulated in response to targeted therapies in multiple cancers, including lung, breast and prostate, and is a mechanism of treatment of resistance. Levels of HER3 expression are highly dynamic, making quantification by tissue sampling techniques such as biopsy potentially challenging and biopsy characterization of all individual metastatic lesions impossible. PET imaging, which permits global and serial imaging may present a more robust method for HER3 quantification, improving HER3 diagnosis and facilitating improved clinical trials assessing HER3 expression, and in the long term providing accurate diagnosis of changes in HER3 status to help identify targeted therapy resistance. In order to develop a PET imaging agent with rapid pharmacokinetics and high in vivo target correlation, phage display was performed to select for a HER3 peptide. The consensus peptide sequence was analyzed for HER binding and *in vitro* and *in vivo*, and binding was correlated with protein expression to determine specificity.

**Methods:** A phage display library bearing cysteine constrained 7 amino acid peptides was selected against the extracellular domain of HER3. Individual phages from the selection were screened by ELISA to determine a candidate peptide. The peptide was characterized for HER3 affinity, specificity and cell binding *in vitro*, prior to *in vivo* PET imaging and biodistribution. The <sup>68</sup>Ga-NOTA peptide was injected into mice bearing high and low HER3 expressing tumors, and PET images were acquired followed by tumor and relevant organ excision for biodistribution and protein correlation.

**Results:** The phage display selection resulted in convergence upon a single peptide, HER3P1, which was demonstrated to bind to HER3 with an affinity of 270±151 nM. A biotinylated peptide exhibited greater than 10-fold specificity over similar receptor tyrosine kinases EGFR and HER2, and could differentiate HER3 expression in cells using multiple fluorescent analyses. The HER3P1 was conjugated to NOTA and radiolabeled with <sup>68</sup>Ga at high purity and specific activity and tested for uptake in HER3-positive and negative tumors. HER3P1 demonstrated excellent tumor to background ratios (1.59-3.32) in HER3+ tumors, which were significantly higher than the low HER3 expressing control tumors (0.84-0.93, P <0.01). Furthermore, *ex vivo* analysis of imaged tumors revealed a high (P < 0.001) correlation between HER3 peptide uptake and both HER3 protein expression and percent injected dose per gram. The peptide was cleared renally, with minimal non-specific accumulation in other non-target organs.

**Conclusion:** A novel, first-in-class HER3 peptide was selected by phage display. The peptide was highly specific for HER3 *in vitro* and *in vivo*, and demonstrated quantitative PET imaging of HER3 expression. These results indicate that HER3P1 represents a promising, clinically translatable HER3 imaging agent, and future translational efforts are planned.

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