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Synthesis and comparative evaluation of novel ^{64}Cu-labeled high affinity cell-specific peptides for positron emission tomography imaging of tumor vasculature

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ABSTRACT

Tumor angiogenesis, the formation of new tumor blood supply, has been recognized as a hallmark of cancer and represents an important target for clinical management of various angiogenesis-dependent solid tumors. Previously, by screening a bacteriophage peptide library we have discovered the FHT-peptide sequence that binds specifically to bone marrow-derived tumor vasculature with high affinity. Here in an effort to determine the potential of the FHT-peptide for in vivo positron emission tomography (PET) imaging of aggressive tumor vasculature we studied four FHT-derivatives: NOTA-FHT, NOTA-(FHT)2, NOTA-PEG-FHT, and NOTA-PEG-(FHT)2. These peptide analogs were synthesized, labeled with the PET radionuclide ^{64}Cu, and characterized side-by-side with small animal PET and computed tomography imaging (microPET/CT) at 1 h, 4 h, and 24 h post injection in a subcutaneous Lewis lung carcinoma (LLC) tumor model. Because of its excellent in vivo kinetic properties and high tumor-to-background ratio, the ^{64}Cu-NOTA-FHT radiopeptide was selected for more detailed evaluation. Blocking studies with excess of unlabeled peptide showed specific and peptide mediated ^{64}Cu-NOTA-FHT tumor uptake. Biodistribution experiments in the same tumor model confirmed microPET/CT imaging results. Human radiation absorbed dose extrapolated from rodent biodistribution of ^{64}Cu-NOTA-FHT revealed favorable dosimetry profile. The findings from this investigation warrant further development of ^{64}Cu-NOTA-FHT as a potential targeted diagnostic radiopharmaceutical for PET imaging of aggressive tumor vasculature.

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1. Introduction

Biomaterials play an essential role in a variety of diagnostic and therapeutic procedures and perform functions to direct and control interactions with living systems that have crucial effects on overall biological performance and clinical outcomes of these procedures [1]. In most instances highly specific interactions have been regarded as key features in biomaterials design improving biocompatibility and recapitulating material-cell specific interactions [2,3]. For example, the short Arg-Gly-Asp (RGD) tripeptide, the minimal sequence for binding to integrin receptors, has been widely utilized to impart biological function to synthetic materials and facilitate material-cell recognition events [4–9]. With recent advances in combinatorial, high throughput screening technologies such as bacreriophage (phage) display, new engineered peptides have been discovered that bind with high affinity to their biologic targets in a highly specific manner [10–14]. Phage display-selected, disease-specific peptides hold promise in precision medicine for use as molecularly targeted diagnostic imaging agents [15–21]. Disease-specific, peptide-based imaging compositions have the potential to extend the clinical utility of conventional

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imaging techniques such as positron emission tomography (PET) by enabling integration of accurate diagnostics with therapy to achieve better outcomes.

Tumor angiogenesis, the formation of new tumor blood supply, is a hallmark of cancer and is generally associated with aggressive tumor progression and poor patient prognosis. Tumors form new blood capillaries either from pre-existing mature ones or *de novo* by recruiting circulating pro-angiogenic endothelial and hematopoietic precursor cells mobilized from the host bone marrow [22]. Stromal cells of bone marrow origin have been identified in the vasculature of several pre-clinical models [23–26]. In humans, bone marrow-derived endothelial cells have been detected in patients with multiple myeloma [27], breast cancer [28], non-small cell lung cancer [29], and malignant gliomas [30]. Increased levels of immature precursors in the peripheral blood of patients with breast, colon, prostate, head and neck, renal and ovarian cancer have been shown to correlate with aggressive disease [31]. Furthermore, circulating progenitor cells of bone marrow origin have been exploited as a potential biomarker to guide the use of antiangiogenic therapy in cancer patients [32]. Together, these studies suggest that bone marrow-sourced, circulating pro-angiogenic tumor-homing cells actively participate in tumor angiogenesis and represent a promising target for development of novel diagnostic and therapeutic agents with improved tumor selectivity.

Screening of phage display random peptide libraries has emerged as useful and practical approach for the discovery of new peptide ligands that can bind with high affinity and specificity to a variety of targets including angiogenic blood vessels [33]. Innovative methodological selection protocols in the environment of the whole mouse have been developed to allow for improved targeting in vivo [14,34]. Besides the optimized pharmacokinetics and in vivo binding affinity, peptides selected by unbiased phage screens typically display specificity of 10–100 fold for their target over background tissue [11,13]. Because phage display-selected ligands possess suitable pharmacokinetic properties, i.e., high affinity, tumor uptake and specificity, these targeting vectors have been actively pursued as agents for molecular imaging and non invasive tumor phenotyping [10–12,14–19,33]. In addition to good transport properties, and the ability to both penetrate rapidly into solid tumors and recognize hidden or rare epitopes, peptides have in general low toxicity and immunogenicity, and can be produced in large quantities at low cost.

By screening a phage display dodecapeptide library in vivo, we have previously discovered a new amino acid sequence, the FHT-peptide (Phe-His-Thr-Pro-Ser-Gln-Asn-Ser-Ala-Phe-Arg-Leu), that specifically binds with high affinity to bone marrow-derived circulating tumor-homing cells [14]. Unlike the RGD sequence, widely utilized to impart specificity to integrin receptors expressed both on tumor neovasculature and tumor cells, the FHT-peptide selectively binds to a protein expressed on the surface of bone marrow-derived neovascular cells. This unique binding profile of the FHT-peptide provides a novel platform for developing more specific diagnostic and therapeutic agents. Here, we will test the ability of the FHT-peptide, labeled with the ^{64}Cu ($t_{1/2}$ = 12.7 h) positron emitting radionuclide, to selectively target tumor vasculature for in vivo PET imaging. Due to its dual decay characteristics ^{64}Cu (β^+: 17.8%, $E_{\beta+max}$ = 653 keV; β^-: 38.4%, $E_{\beta-max}$ = 578 keV) has promising clinical applications both for diagnostic imaging and targeted radiotherapy. Positron emissions from a ^{64}Cu-labeled peptide radiopharmaceutical will allow the oncologist to obtain diagnostic PET image assessing the malignancy and then provide personalized treatment to the patient using the same ^{64}Cu-radiolabeled composition.

The goal of this study is to identify an FHT-based molecular composition for a PET imaging agent providing the highest ratio of tumor uptake to normal tissue uptake in vivo. Here we report studies that explore the pharmacokinetics and imaging characteristics of four distinct molecular constructs based on the FHT-sequence (Fig. 1) all labeled with the ^{64}Cu radionuclide for PET imaging. FHT-derivatives were prepared with the ^{64}Cu-binding chelate 1,4,7-triazacyclononane 1,4,7-triacetic acid (NOTA). Modifications such as PEGylation and divalent ligand presentation were utilized to modulate in vivo distribution properties of the FHT-compositions. Both approaches, PEGylation and multivalency, are known to modify in vivo pharmacokinetics of small molecules [16,20,21]. FHT-peptide derivatives were prepared using solid phase peptide synthesis protocols, radiolabeled with ^{64}Cu, and characterized side-by-side using dual small animal PET and computed tomography (CT) imaging (microPET/CT) at 1 h, 4 h, and 24 h post injection in a subcutaneous Lewis lung carcinoma (LLC) tumor model. Biodistribution experiments were performed in the same tumor system. Peptide-specific uptake of the lead FHT-composition was demonstrated in blocking studies. Human absorbed doses for the selected lead construct were extrapolated from the rodent biodistribution.

2. Materials and methods

2.1. Reagents and analyses

All chemicals and reagents were of analytical grade or better and were used without further purification. Rink Amide-ChemMatrix resin (loading, 0.52 mmol/g) was purchased from PCAS BioMatrix (Quebec, Canada). 9-Fluorenylmethyloxycarbonyl (Fmoc)-protected standard amino acids, Fmoc-Lys(Fmoc)-OH, Fmoc-Lys(Mtt)-OH, di-tert-butyl dicarbonate (Boc_2O), and the coupling agent 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b] pyridinium 3-oxid hexafluorophosphate (HATU) were purchased from AnaSpec (Freemont, CA). Fmoc-NH-$(PEG)_{12}$-$CH_2CH_2CO_2H$ [1-(9H-fluoren-9-ylmethoxycarbonylamino)-3,6,9,12,15,18,21,24,27,30,33,36-dodecaoxanonatria contan-39-oic acid] was purchased from Quanta Biodesign (Plain City, OH) and p-SCN-Bn-NOTA (2-S-(4-Isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid) was obtained from Macrocyclics (Dallas, TX). All other solvents and chemicals were purchased from VWR (Atlanta, GA).

The solid phase peptide synthesis was performed on Rink Amide-ChemMatrix resin using PTI Symphony (Protein Technologies, Tuscon, AZ) peptide synthesizer. Analytic high-performance liquid chromatography (HPLC) analyses were performed on a Waters Alliance HPLC system and semi-preparative HPLC purifications were conducted on a Waters 1525 HPLC system. Matrix-assisted laser desorption and ionization time of flight mass spectrometry (MALDI-TOF MS) analysis was carried out using an α-cyano-4-hydroxycinnamic acid matrix on an ABI 4800 mass spectrometer (Sciex, Framingham, MA).

High specific activity ^{64}Cu was obtained from the Washington University School of Medicine (St. Louis, MO) as $^{64}CuCl_2$ in 0.5 M HCl. Copper-64 was produced on a CS-15 biomedical cyclotron by the ^{64}Ni(p,n)^{64}Cu nuclear reaction using previously published methods [35]. All experiments involving the use of radioactive materials were conducted under the authorization of the Radiation Safety Committee at the University of North Carolina – Chapel Hill.

2.2. Peptide synthesis and characterization

Monomeric peptide derivatives were synthesized using 20 μmol of the resin, and dimeric peptide derivatives were synthesized using 12 μmol of the resin. Fmoc-Lys(Mtt)-OH, Fmoc-Lys(Fmoc)-OH,

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