Preparation and Evaluation of ZD2 Peptide $^{64}$Cu-DOTA Conjugate as a Positron Emission Tomography Probe for Detection and Characterization of Prostate Cancer

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ABSTRACT: Positron emission tomography (PET) is a sensitive modality for cancer molecular imaging. We aim to develop a PET probe for sensitive detection and risk stratification of prostate cancer by targeting an abundant microenvironment oncoprotein, extradomain-B fibronectin (EDB-FN). The probe consists of a small ZD2 peptide specific to EDB-FN and a $^{64}$Cu-DOTA chelate. The probe was synthesized using standard solid-phase peptide chemistry and chelated to $^{64}$Cu prior to imaging. PET images were acquired at 4 and 22 h after intravenously injecting a 200 μCi probe into mice bearing human PC3 and LNCaP tumors, which represent highly aggressive and slow-growing prostate tumors, respectively. At 4 and 22 h postinjection, tumors could be clearly identified in the PET images. A significant higher signal was observed in PC3 tumors than in LNCaP tumors at 22 h ($p = 0.01$). Probe accumulation was also higher in PC3 tumors at 24 h. These data demonstrated that PET molecular imaging of EDB-FN in the tumor microenvironment of prostate cancer allows efficient differentiation of PC3 and LNCaP tumors in vivo. The ZD2 peptide-targeted PET probe shows potential in the detection and characterization of high-risk prostate cancer to improve the clinical management of prostate cancer.

INTRODUCTION

Prostate cancer is a prevalent disease with a high heterogeneity in men over 50 years of age. The current diagnostic approaches for prostate cancer, such as prostate-specific antigen screening, have a high false-positive rate, leading to overdiagnosis and overtreatment.1 A noninvasive approach that can accurately detect prostate cancer and stratify the risk of the disease is highly valuable for tailoring precision therapies for patients who need immediate intervention and spare those with low-risk tumors. Positron emission tomography (PET) is a highly sensitive molecular imaging modality routinely used in clinical management of cancer. The development of PET probes has mostly been focused on targeting the markers associated with overexpressed cell receptors3 and elevated metabolisms.4 However, the expression levels of these markers are subject to change during disease progression, and a single biomarker cannot always suffice for cancer diagnosis because of cancer heterogeneity.5 Tumor microenvironment plays a critical role in the outgrowth, migration, and metastasis of aggressive tumors.6 The tumor microenvironment biomarkers, including angiogenesis, hypoxia, acidic pH, extracellular matrix (ECM), and so forth, are critical determinants of tumor aggressiveness regardless of the heterogeneity of cancer cells. Molecular imaging of these tumor microenvironment biomarkers could be a promising approach for accurate detection and characterization of aggressive cancers, including prostate cancer.7,8

Among the ECM proteins, extradomain-B fibronectin (EDB-FN) is considered as one of the most cancer-selective proteins with negligible expression in normal adult tissues.9 The upregulation of oncofetal fibronectin, including EDB-FN, is an important hallmark of epithelial-to-mesenchymal transition (EMT).10,11 EMT plays an important role in the invasion, metastasis, and drug resistance of many malignant human cancer types, including prostate cancer.12 It has been demonstrated clinically that EDB-FN is highly expressed in high-risk prostate cancer, but lowly expressed in benign lesions and benign prostatic hyperplasia.13−16 Further, EDB-FN expression is inversely correlated with patient survival.10,17−19 Therefore, EDB-FN is a promising molecular imaging target for early detection and risk stratification of prostate tumors. We have recently developed targeted magnetic resonance imaging (MRI) contrast agents specific to EDB-FN and...
demonstrated the potential of molecular imaging of the ECM protein for cancer detection and risk stratification in mouse models of breast and prostate cancer. These contrast agents were constructed by conjugating a small-peptide ZD2 (Thr-Val-Arg-Thr-Ser-Ala-Asp) to gadolinium chelates and a gadofullerene. As compared to antibody-labelled probes, the small-peptide-targeted imaging agents possess the advantages of better tumor-penetrating ability and minimal background noise because of rapid clearance of unbound agents from the circulation for clear delineation of aggressive tumors. The development of ZD2-targeted PET probes has the potential for highly sensitive and quantitative molecular imaging of EDB-FN for accurate detection and risk stratification of aggressive tumors.

Herein, we aim to develop a ZD2 64Cu-DOTA conjugate as a PET probe for EDB-FN and evaluate its efficacy for PET imaging in mice bearing aggressive PC3 and slow-growing LNCaP human prostate tumor xenografts. Previously, we showed that EDB-FN was highly expressed in aggressive PC3 tumors and negligibly expressed in slow-growing and nonmetastatic LNCaP tumors. MRI with a EDB-FN targeted contrast agent ZD2-Gd(HP-DO3A) showed stronger contrast enhancement in the PC3 tumors than in the LNCaP tumors. The use of 64Cu is particularly attractive because of its 12.74 h half-life, providing extended imaging time frame for cancer detection in the prostate with minimal background inference, especially from the bladder. The PET probe was synthesized by conjugating the ZD2 peptide to a macrocyclic chelate DOTA using solid-phase peptide chemistry, followed by complexation with 64CuCl2 (Figure 1). A short spacer with two repeats of 8-amino-3,6-dioxaoctanoic acid was introduced between the peptide and the chelator. The targeted ligand ZD2-DA-DOTA was purified by preparative high-performance liquid chromatography (HPLC) and characterized by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry $m/z = 1425.8$ (M + 1), observed; 1425.5, calculated]. The preparation of the targeted PET probe was demonstrated by complexation of equal molar ZD2-DA-DOTA in phosphate-buffered saline (PBS) buffer (pH 7.4) and cold CuCl2 in dilute HCl (0.1 N) at 45 °C for 30 min, the same condition used for radiolabeling. The formation of ZD2-DA-(Cu-DOTA) was verified by MALDI-TOF mass spectrometry $m/z = 1487.8$ (M + 1), observed; 1486.04, calculated].

The efficacy of ZD2-DA-(64Cu-DOTA) for prostate cancer PET imaging was then investigated in male nude mice bearing both PC3 and LNCaP human prostate cancer xenografts. Previously, we showed that EDB-FN was highly expressed in aggressive PC3 tumors and negligibly expressed in slow-growing and nonmetastatic LNCaP tumors. The tumor models were used to represent high-risk and low-risk prostate tumors and to test the ability of the probe to detect and stratify aggressive prostate cancer. Radiolabeling was performed by mixing 20 μL of 64Cu(II) solution (0.1 N HCl, ca. 1 mCi or 37 MBq) with 480 μL of ZD2-DA-DOTA (0.05 mg/mL, a large excess, PBS, pH = 7.4) in a 1.5 mL microcentrifuge tube and was maintained at 45 °C for 30 min with intermittent shaking. The reaction mixture was then diluted in the ratio of 1:2 with PBS and tested with a pH paper to ensure neutral pH for
intravenous injection. The radiotracer was injected intravenously at the dose of 7.4 MBq (200 μCi) per mouse. PET images of the mice were acquired in a group of four mice bearing both PC3 and LNCaP tumor xenografts at 4 and 22 h after the injection.

Figure 2 shows the representative three-dimensional (3D) volume rendering and axial PET/computed tomography (CT) images of two tumor-bearing mice at 4 and 22 h after injection of ZD2-DA-(64Cu-DOTA). Stronger signal was visible in the aggressive PC3 tumors than in the slow-growing LNCaP tumors. The location and size of PC3 tumors were clearly delineated in the PET images. The tracer uptake or signal intensity was quantitatively analyzed in the region of interest (ROI) at 4 and 22 h. As shown in Figure 3, ZD2-DA-(64Cu-

Figure 2. Macroscopic bright-field and 3D volume rendering PET/CT images of two representative mice bearing LNCaP and PC3 tumors at 4 and 22 h after injection of ZD2-DA-(64Cu-DOTA). Stronger signal was visible in the aggressive PC3 tumors than in the slow-growing LNCaP tumors. The location and size of PC3 tumors were clearly delineated in the PET images. The tracer uptake or signal intensity was quantitatively analyzed in the region of interest (ROI) at 4 and 22 h. As shown in Figure 3, ZD2-DA-(64Cu-DOTA) resulted in higher probe uptake in PC3 tumors than in LNCaP tumors. At 22 h, PET revealed an over two-folds higher accumulation of PET tracer in highly aggressive PC3 tumors (7711 ± 1994 Bq/mL) compared to the less aggressive LNCaP tumors (3213 ± 1511 Bq/mL) (N = 4, P < 0.05, two-tailed Student’s t test), which corroborated that the probe preferentially accumulates in the more aggressive PC3 tumor than in the nonmetastatic LNCaP tumor.

The biodistribution of the radiotracer was measured after euthanizing the mice at 24 h postinjection (Figure 4. The biodistribution pattern was consistent with the findings in PET imaging, with a strong uptake in the tumors, liver, and renal pathways. The biodistribution of the radiotracer was measured after euthanizing the mice at 24 h postinjection (Figure 4). The biodistribution pattern was consistent with the findings in PET imaging, with a strong uptake in the tumors, liver, and kidney. Other organs, such as brain and muscle, exhibited a low radiotracer uptake, which is a desirable property of the

Figure 3. Quantitative tracer uptake in the muscle, liver, heart, and LNCaP and PC3 tumors at 4 and 22 h after ZD2-DA-64Cu(DOTA) injection (N = 4).

Figure 4. Biodistribution of ZD2-DA-(64Cu-DOTA) in different tissues at 24 h after injection. Data is presented as mean ± sem (N = 3).

The expression of EDB-FN in the prostate tumors was determined by immunofluorescence staining of the tissue section of PC3 and LNCaP tumors with an anti-EDB-FN monoclonal antibody BC1 after PET imaging. An Alexa Fluor 488-conjugated anti-mouse antibody was used to stain the BC1 antibody and EDB-FN. Figure 5 shows the fluorescence images of the tumor sections acquired with an Olympus FV1000 confocal laser scanning microscope. Strong fluorescence staining was visible in the PC3 tumor section, whereas little staining was observed in the LNCaP tumor. Consistently, we have previously shown that the EDB mRNA level in LNCaP cells was lower than that in PC3 cells. The EDB-FN expression levels in two different prostate tumors correlated well with the observation with PET molecular imaging. The result suggests that ZD2-DA-(64Cu-DOTA) is effective for sensitive and quantitative visualization of EDB-FN expression in prostate cancer.

**DISCUSSIONS**

PET imaging has been applied in the clinical examination of prostate cancer mainly with [18F]-FDG, based on elevated glucose metabolism of prostate cancer compared to that of...
normal tissues. However, [18F]-FDG PET has not demonstrated the ability to differentiate benign prostate cancer from aggressive ones. PSMA-specific PET probes have recently been developed for prostate cancer. Clinical studies have demonstrated the ability of the PSMA probes for effective detection of PSMA-positive prostate tumors. However, a recent study cautioned that the PSMA probes may not be able to differentiate benign tissues from prostate cancer. Novel PET probes are needed to detect and risk-stratify aggressive prostate cancer to meet the clinical need of a noninvasive diagnostic modality for precision clinical management of prostate cancer.

We have shown in this study the potential of PET imaging of the ECM oncoprotein EDB-FN with a peptide probe ZD2-DA-(64Cu-DOTA) for detection and characterization of prostate cancer. Previously, we have shown that EDB-FN is highly expressed in the fast-growing PC3 tumors and lowly expressed in the slow-growing LNCaP tumors. ZD2 peptide-targeted MRI contrast agents were able to generate strong signal enhancement in PC3 tumors than in LNCaP tumors. The results of PET molecular imaging EDB-FN with ZD2-DA-(64Cu-DOTA), especially at 22 h postinjection, are in agreement with MR molecular imaging with a ZD2 peptide-targeted MRI contrast agent. When comparing the probe uptake in the tumors, stronger PET signals were detected in the fast-growing PC3 tumors with a high EDB-FN expression than in the slow-growing LNCaP tumors. However, a significant signal intensity was still observed in the LNCaP tumors of the PET images. This could be attributed to the relatively low chelation stability of 64Cu-DOTA monoamide. It has been shown that free 64Cu(II) released from the chelate could accumulate in the prostate tumors in animal models.

The relatively high signal intensity in the LNCaP tumors could be attributed to the accumulation of free 64Cu(II) released from the probe. Nevertheless, the targeting effect of the ZD2 peptide of the probe still resulted in significantly higher signal intensity in the PC3 tumors than in the LNCaP tumors. As compared to MR molecular imaging, PET imaging produces sensitive and quantitative visualization and measurement of EDB-FN expression levels in prostate cancer, which could provide more accurate risk stratification of aggressive prostate cancer.

Generally, PET imaging with probes of relatively short half-lives suffers from significant signal inference from the bladder for imaging primary tumors in the prostate because of a limited imaging window. The relatively long half-life 64Cu allows sufficient time to empty the bladder and to minimize the potential signal interference from the bladder, which is critical for early detection of primary tumors in the prostate. Substantial signals were still visible in tumors at 22 h postinjection with little signals in the bladder. Significant signal intensity was observed in the liver with ZD2-DA-(64Cu-DOTA), which could also be attributed to the relatively low stability of Cu-DOTA monoamide. The release of free 64Cu(II) from the chelate may lead to nonspecific accumulation of the radioisotope in the liver.

Antibodies and antibody fragments have been developed to target EDB-FN for the detection of cancer, including prostate cancer. A recent clinical study has shown that an antibody-labelled PET probe, 131I-L19-SIP, specific to EDB-FN was effective for imaging advanced prostate cancer with PET, suggesting that EDB-FN is a promising molecular marker for clinical PET imaging of prostate cancer. This study has shown that the small-peptide-targeted PET probe specific to EDB-FN also has the potential for prostate cancer imaging. As compared to antibody-based probes, small-peptide PET probes may possess several advantages, including cost-effective production, better tumor penetration through diffusion and perfusion, and rapid excretion of the unbound probe from circulation.

Further optimization is needed to improve the stability of ZD2-targeted PET probes. We will choose ligands that form stable complexes with 66Cu(II), which will minimize the release of free 66Cu(II) ions and the consequent nonspecific tissue uptake. The clinically used radioisotopes, for example, 68Ga, will be used to develop the ZD2-targeted PET probe for sensitive detection and risk stratification of prostate cancer. The specific overexpression of EDB-FN has also been reported in a number of human cancers, including melanoma, breast cancer, glioma, head and neck cancer, and so forth. An optimized ZD2 peptide-targeted PET probe has the potential to be used for imaging of a broad spectrum of human cancers. Currently, we are working on the optimization of ZD2-targeted PET probes and testing the ability of the probes for imaging other types of cancer.

### CONCLUSIONS

A small-peptide-targeted PET probe, ZD2-DA-64Cu(DOTA), specific to EDB-FN was synthesized and tested for prostate cancer imaging. The probe was effective in detecting prostate tumors and exhibited higher uptake in high-risk prostate tumors with a high EDB-FN expression than in low-risk tumors in animal models. The results suggest that ZD2-targeted PET probes have the promise for sensitive detection and characterization of prostate cancer. Optimizations of the targeted probe are needed to improve the chelation stability of the radioisotope and the ligand to minimize the nonspecific uptake in a normal tissue and to further improve its accuracy for quantitative determination of the EDB-FN expression and risk stratification of aggressive prostate cancer. The high sensitivity of PET and specific detection and characterization of the targeted probe provide another toolset for prostate cancer diagnosis and precision management of the disease.

### MATERIALS AND METHODS

#### Synthesis of ZD2-DA-DOTA and Chelates.

The reagents used for chemical synthesis were purchased from Sigma-Aldrich (Saint Louis, MO, USA), unless otherwise stated. Fmoc-protected amino acids and 2-chlorotrityl chloride resin were acquired from Chem-Impex International, Inc. (Wood Dale, IL). The spacer, Fmoc-8-amino-3,6-dioxoacetic acid (Fmoc–NH–(CH2)3CH2O–CH2COOH), was acquired from Chempep (Wellington, FL). 1,4,7,10-Tetraazacyclododecane-1,4,7-tris- tert-butyl acetate-10-acetic acid (DOTA-tris(t-Bu)) was purchased from TCI America (Portland, OR).

The precursor ZD2-DA-DOTA, which contains the ZD2 peptide (sequence: TVRTSD), two repeats of NH2–(CH2)3CH2O–CH2COOH, and DOTA was synthesized by sequentially adding the corresponding protected amino acids, Fmoc–NH–(CH2)3CH2O–CH2COOH, and t-Bu-DOTA on the resin in a solid phase using standard Fmoc-peptide chemistry. The product was then cleaved off the resin using trifluoroacetic acid/triisopropyl silane/H2O (96.5:1:2.5) and stirred at room temperature for 3 h and precipitated in ether to
give a crude product. The final product was purified using preparative HPLC on an Agilent 1100 HPLC system equipped with a semipreparative C18 column (Agilent Technologies, Santa Clara, CA). ZD2-DA-DOTA was characterized by MALDI-TOF mass spectrometry on a Voyager DE-STR spectrometer (PerkinElmer, Waltham, MA) in the linear mode with R 2,5-dihydroxybenzoic acid as a matrix (M + 1: 1425.8, observed; 1425.7, calculated).

**Cell Culture and Animal Models.** The animal study has been approved by the Institutional Animal Care and Use Committee of the Case Western Reserve University (CWRU), and all subjects signed an informed consent form. PC3 and LnCaP cells were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Roswell Park Memorial Institute medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a humid incubator maintained at 37 °C and 5% CO2. Male athymic nude mice (4–6 weeks old) were acquired from the Case Comprehensive Cancer Center (Cleveland, OH, USA) and housed in the CWRU Animal Care Facility. Three million cells in high concentration Matrigel (Corning, Tewksbury, MA) were used for tumor inoculation. LnCaP cells were subcutaneously inoculated in the left flank of the mice. Four weeks later, PC3 cells were inoculated on the right flank of the same mice for PET imaging.

**Radiolabeling.** The radioisotope 64Cu(II) was acquired from the University of Wisconsin–Madison (Madison, WI). The chelation of ZD2-DA-DOTA with Cu(II) was first tested with cold CuCl2 in 0.1 N HCl aqueous solution under the same condition as radiolabeling. Equal molar ZD2-DA-DOTA in PBS buffer (pH 7.4) and CuCl2 solution was mixed and stirred at 45 °C for 30 min. The formation of ZD2-DA-(Cu-DOTA) was verified by MALDI-TOF mass spectrometry (M + 1: 1487.8, observed; 1486.04, calculated). For radiolabeling, 10 μCi 64Cu(II) was dissolved in 200 μL of 0.1 N HCl. Twenty microliters of 64Cu(II) solution (ca. 1 mCi) was mixed with 480 μL of ZD2-DA-DOTA (0.05 mg/mL, a large excess, PBS) in a 1.5 mL microcentrifuge tube. The vessel was then maintained by heating at 45 °C for 30 min with intermittent shaking. The final pH of the solution was adjusted to be neutral using NaOH solution before injection.

**PET Imaging.** All in vivo imaging studies were conducted according to the CWRU Animal Research Committee-approved protocols and guidelines. The mice were anesthetized with 2% isoflurane in oxygen and injected with ~200 μCi (~7.4 MBq) ZD2-DA-64Cu(DOTA) via the tail vein. The mice underwent 10 min static PET scans after 4 and 22 h uptake period PET scans (Inveon microPET, Siemens Medical Solutions USA Inc.). Images were reconstructed using 3D-OSEM with 3D histogramming and a zoom factor of 1.0 (two iterations followed by MAP with 18 iterations). CT scans (Siemens Medical Solutions USA Inc.) were performed after PET procedures for anatomical coregistration. AMIDE version 1.5.557 and AMIRA software were used to analyze the PET/CT images g. ROIs were drawn for PC3 and LnCaP tumors to calculate the ratio of specific to nonspecific (muscle) binding.

**Biodistribution.** After the last micro-PET/CT imaging at 22 h postinjection, three mice were euthanized, the organs and blood were collected and weighed, and the activity was determined in a gamma counter. The percent-injected dose per gram of tissue was calculated using a standard containing 2% of the injected dose.

**Histological Analysis.** After image acquisitions, the mice were euthanized. The tumors were harvested, embedded in an optimal cutting temperature medium, frozen in −80 °C, cryosectioned at 5 μm, and permeabilized with cold acetone. The tissue was blocked with bovine serum albumin (1%) in PBS at room temperature for 1 h. Anti-EDB-FN BC1 antibody (Abcam, Cambridge, MA) was incubated with the tissue section of PC3 and LnCaP tumors. After extensive washing, secondary anti-mouse Alexa Fluor 488 antibody was incubated for 1 h. Tissue sections were counterstained with Prolong Gold antifade mounting medium with 4′-6-diamidino-2-phenylindole (Thermo Fisher, Waltham, MA). The stained tissues were imaged on an Olympus FX1000 confocal laser scanning microscope.

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**Notes**

The authors declare the following competing financial interest(s): ZRL is one of the founders of Molecular Theranostics, LLC and Motek Pharmaceuticals, which are focused on the commercialization of targeted imaging agents. ZRL, SQG and YL have ownership interest in the companies.

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