Nuclear Medicine and Biology 43 (2016) 247–252

Contents lists available at ScienceDirect

Nuclear Medicine and Biology

journal homepage: www.elsevier.com/locate/nucmedbio

^{18}F-nanobody for PET imaging of HER2 overexpressing tumors

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ARTICLE INFO

Article history:
Received 23 July 2015
Received in revised form 15 October 2015
Accepted 20 January 2016

Keywords:
Nanobodies
[^{18}F]-SFB
HER2
PET
Molecular imaging

ABSTRACT

Introduction: Radiolabeled nanobodies are exciting new probes for molecular imaging due to high affinity, high specificity and fast washout from the blood. Here we present the labeling of an anti-HER2 nanobody with ^{18}F and its validation for *in vivo* assessment of HER2 overexpression.
Methods: The GMP grade anti-HER2 nanobody was labeled with the prosthetic group, N-succinimidyl-4-[^{18}F] fluorobenzoate ([^{18}F]-SFB), and its biodistribution, tumor targeting and specificity were evaluated in mouse and rat tumor models.
Results: [^{18}F]FB-anti-HER2 nanobody was prepared with a 5–15% global yield (decay corrected) and a specific activity of 24.7 ± 8.2 MBq/nmol. *In vivo* studies demonstrated a high specific uptake for HER2 positive xenografts (5.94 ± 1.17 and 3.74 ± 0.52%IA/g, 1 and 3 h p.i.) with high tumor-to-blood and tumor-to-muscle ratios generating high contrast PET imaging. The probe presented fast clearance through the kidneys (4%IA/g at 3 h p.i.). [^{18}F]FB-anti-HER2 nanobody is able to image HER2 expressing tumors when co-administered with the anti-HER2 therapeutic antibody trastuzumab (Herceptin), indicating the possibility of using the tracer in patients undergoing Herceptin therapy.
Conclusions: The GMP grade anti-HER2 nanobody was labeled with ^{18}F. This new PET probe for imaging HER2 overexpression in tumors has ample potential for clinical translation.

1. Introduction

Nanobodies (nbs) are the single-domain antigen binding fragments derived from camelid heavy-chain-only antibodies [1,2]. Their small size (12–15 kDa), high stability, nanomolar affinity and low immunogenicity make them excellent probes for molecular imaging [2–4] and recently their potential for targeted radionuclide therapy has also been evaluated [5–9]. Radiolabeled nbs target their antigen fast and efficiently *in vivo* and are rapidly eliminated from non-target organs (except kidneys), generating high contrast images at early time points after probe injection, thus enabling functionalization of nbs with short lived radioisotopes [2,3]. Nanobody based nuclear tracers have been developed against different targets in oncology (human epidermal growth factor receptor type 2 (HER2), epidermal growth factor receptor (EGFR), carcinoembryonic antigen (CEA), prostate-specific membrane antigen (PSMA), multiple myeloma 5 T2 M-protein) [9–18], inflammation (macrophage mannose receptor (MMR), V-set and immunoglobulin domain containing 4 (VSIG4)) [19–21] and cardiovascular disease (vascular cell adhesion molecule 1 (VCAM1)), Lectin-like oxidized low-density lipoprotein receptor (LOX1) [22–24].

HER2 is overexpressed in about 20% of breast, 15–30% of gastric and 9–32% of ovarian cancer and studies have demonstrated that HER2 overexpression is associated with increased tumor aggressiveness and higher relapse and mortality rates [25–27]. In this way patients can profit from a HER2-targeted therapy treatment (e.g. monoclonal antibodies trastuzumab and pertuzumab, trastuzumab-drug conjugate T-DM1, Lapatinib), which improves survival significantly. Currently, HER2 status is determined at diagnosis by immunohistochemistry (IHC) and gene analysis (e.g. Fluorescence in Situ Hybridization (FISH)) on a biopsy specimen with a chance of false negative results [28,29]. Furthermore studies reported a significant HER2 discordance between primary carcinoma and metastases up to 27% [30–33]. International guidelines therefore now recommend reassessment of HER2 status in the metastatic setting. Given the invasiveness and risks of metastatic biopsy, a non-invasive HER2 assessment using PET/CT imaging could improve patient care. Moreover, imaging will assess all disease locations, thereby overcoming the risk of sampling error and providing information on heterogeneous expression. Radiolabeled nanobodies could be used as imaging probes to assess whole-body HER2 status with a non-invasive PET/CT scan with potential to improve patient

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http://dx.doi.org/10.1016/j.nucmedbio.2016.01.002
0969-8051/

stratification for HER2-targeted therapy, thereby increasing survival benefits while reducing morbidity and treatment toxicity.

A first phase I clinical trial with a radiolabeled nanobody for detection of HER2 overexpression in breast cancer has recently been finalized in the Universitair Ziekenhuis Brussel (UZ Brussel). The lead anti-HER2 nanobody (2Rs15d) has been previously selected based on different criteria including high production yield, target affinity, recognition of an epitope different from that of the HER2 therapeutic monoclonal antibodies trastuzumab and pertuzumab, tumor targeting and high contrast imaging in tumor mice obtained when radiolabeled with ^{99m}Tc [10]. The nanobody format was optimized and produced without hexahistidine (His6) tag because it might induce immune responses [34]. Clinical translation was implemented for a ^{68}Ga-labeled anti-HER2 nanobody, of which the preclinical validation has been previously described [11]. This first clinical trial demonstrates the translational potential of radiolabeled nanobodies. Besides ^{68}Ga-labeled nanobodies there is a high interest in developing ^{18}F-labeled nanobodies for preclinical and clinical evaluation.

^{18}F is a widely used radionuclide for PET imaging in nuclear medicine with advantageous properties: its half-life of 109.8 min, longer than that of ^{68}Ga (67.7 min), allows image acquisition over a longer time window as well as transport of ^{18}F-tracers to other imaging centers. N-succinimidyl 4-[^{18}F]fluorobenzoate ([^{18}F]-SFB) is one of the most commonly used prosthetic groups for fluorination of proteins and peptides. This activated ester is conjugated to the ε-amino group of solvent exposed lysine residues on proteins via an acylation reaction [35–46].

The aim of the present study was to optimize the synthesis of ^{18}F-anti-HER2 nb using [^{18}F]-SFB as prosthetic group and subsequently to determine whether the ^{18}F-anti-HER2 nb probe is suitable for PET imaging of HER2 overexpression. The probe was validated *in vitro* and *in vivo* in HER2-positive tumor xenografted mice and rats.

2. Materials and methods

2.1. Production and purification of nanobodies

The lead anti-HER2 nb 2Rs15d and the non-targeting control nb BcII10 (directed against the β-lactamase Bacillus cereus 569/H) were produced as described previously [10,11].

2.2. Chromatographic analysis

Size-exclusion chromatography (SEC) was performed on a Superdex® 75 5/150 GL column (GE Healthcare) using 0.01 M phosphate buffer and 0.14 M NaCl (PBS), pH 7.4, at a flow rate of 0.3 mL min^{-1}. A polystyrene divinylbenzene copolymer column (PLRP-S 300 Å, 5 μm, 250/4 mm; Agilent) was used for reverse-phase high performance liquid chromatography (RP-HPLC) with the following gradient (A: 0.1% trifluoroacetic acid in water; B: 0.1% trifluoroacetic acid in acetonitrile): 0–5 min, 25% B; 5–7 min, 25%–34% B; 7–10 min, 34%–100% B; and 10–25 min, 100% B, at a flow rate of 1 mL min^{-1}. The analyses were performed on a Merck Hitachi Chromaster system (VWR, Belgium) connected to a diode array detector and γ-detector (Raytest, Germany).

2.3. Radiochemistry

2.3.1. [^{18}F]-SFB Synthesis

N-succinimidyl-4-[^{18}F] fluorobenzoate ([^{18}F]-SFB) was synthesized as previously reported [35]. The synthesis was performed on a SynthERA® module (IBA Molecular, Belgium) using disposable cassettes (IFP™ nucleophilic, ABX, Germany). Briefly, to the dried K_{222}/K[^{18}F]F complex (11–26 GBq), 4 mg (0.011 mmol) of 4-(ethoxycarbonyl)-N,N,N-trimethylbenzenaminium triflate (ABX, Germany) in dimethyl sulfoxide (DMSO, 2 mL) (Sigma-Aldrich, Belgium) was added and the mixture was heated to 110 °C for 15 min to produce ethyl-4-[^{18}F] fluorobenzoate. Then a 0.1 M solution of tetrapropylammoniumhydroxide (TPAOH) (20 μL, 0.02 mol) was added and heated to 95 °C for 5 min. Subsequently 26 mg (0.072 mmol) of N,N,N′,N′-tetramethyl-O-(N-succinimidyl)uronium hexafluorophosphate (HSTU) in acetonitrile (CH_3CN, 1 mL) was added, and the mixture was heated to 110 °C for 5 min. Next, the reaction mixture was diluted with 4 mL 4.8% acetic acid solution and 8 mL 0.9% NaCl solution and transferred to a C-18 cartridge (Waters) for solid phase extraction. After washing with 20% CH_3CN/H_2O (5 mL), [^{18}F]-SFB was eluted with 2.5 mL CH_3CN. The purified [^{18}F]-SFB was transferred to a conical vial, placed in a homemade semi-automatic module connected to the SynthERA® module, and dried by means of gentle heating and nitrogen stream. The purity of the [^{18}F]-SFB was verified by RP-HPLC (t_R = 13.5 min). Other species could also be identified with the used gradient: free [^{18}F]Fluoride t_R = 3–4 min; hydrolysed [^{18}F]-SFB t_R = 11.5 min; other [^{18}F]-SFB degradation products t_R = 8–9 min.

2.3.2. Conjugation of [^{18}F]-SFB to nanobodies

Nb in 0.1 M borate buffer pH 8.4–8.5 (17–19 nmol, 0.8 mg/mL, 300 μL) was added to dried [^{18}F]-SFB (1.9–7.4 GBq) and incubated for 20 min at room temperature. The labeling mixture was purified by size exclusion chromatography using a disposable PD-10 column (GE Healthcare) (pre-equilibrated with 0.01 M PBS 7.4) and passed through a 0.22 μm filter (Millipore, USA). The final solution was analyzed by RP-HPLC (t_R [^{18}F]FB-nb = 12.4 min).

2.3.3. In vitro stability of [^{18}F]FB-2Rs15d

Stability of [^{18}F]FB-2Rs15d was evaluated by adding 9–23 MBq to 300 μL of human plasma or 400 μL PBS, incubated at 37 °C up to 3 h and analyzed using SEC or RP-HPLC.

2.4. Cells and culturing conditions

The human ovarian cancer cell line SK-OV-3, bearing 3×10^6 HER2 cell surface proteins [47, Suppl Fig. 1], was obtained from American Type Culture Collection and cultured as previously described [10].

2.5. Animal model

The ethical committee for animal experiments of the Vrije Universiteit Brussel approved the animal study protocols. Wild type (WT) C57BL/6 mice (Charles River) were used for *in vivo* stability studies. To evaluate biodistribution and tumor uptake, female athymic nude mice (5 weeks old) and female athymic nude rats (7 weeks old) (Charles River) were subcutaneously inoculated in the right hind leg (mice) or in the right shoulder (rats) with SK-OV-3 cells (4×10^6 for mice and 10×10^6 for rats) suspended in PBS, under the control of 2.5% isoflurane in oxygen (Abbott). The tumors were allowed to grow for 6–7 weeks (20–80 mg in mice and 200–500 mg in rats).

2.6. Biodistribution

SK-OV-3 tumor-bearing mice were injected intravenously with [^{18}F] FB-2Rs15d (10.41 ± 1.05 MBq) (n = 4, 5 μg nb) or [^{18}F]FB-BcII10 (6.15 ± 0.23 MBq) (n = 4, 5 μg nb) to evaluate biodistribution 3 h after injection. One group (n = 6) of SK-OV-3 xenografted mice was pretreated with the anti-HER2 monoclonal antibody trastuzumab (300 μg), 72 h before injection of [^{18}F]FB-2Rs15d (8.68 ± 0.28 MBq; 5 μg nb) to evaluate competition. This group of animals was sacrificed at 1 h post injection (p.i.) and compared to the group without pretreatment (n = 6). All injections were performed *via* the tail vein under the control of 2.5% isoflurane in oxygen.

Animals were dissected and organ activities were counted against a standard of known activity with a gamma-counter (Cobra II inspector 5003, Canberra-Packard) and expressed as percentage of injected activity per gram of tissue (%IA/g), corrected for decay.