Preclinical evaluation of [^{18}F]cabozantinib as a PET imaging agent in a prostate cancer mouse model

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ABSTRACT

Introduction: Cabozantinib is a tyrosine kinase inhibitor (TKI) approved for the treatment of medullary thyroid cancer, renal cell carcinoma and hepatocellular carcinoma, and is currently in clinical trials for the treatment of prostate cancer and others. It exerts its therapeutic effect mainly through inhibition of the tyrosine kinases MET (hepatocyte growth factor receptor) and VEGFR2 (vascular endothelial growth factor receptor 2), in addition to several other kinases involved in cancer. PET imaging with TKIs such as [^{18}F]cabozantinib could potentially aid in cancer diagnosis and guide treatment. This study aims to evaluate the utility of [^{18}F]cabozantinib as a PET imaging probe in PC3 tumor xenografted mice.

Methods: [^{18}F]cabozantinib was evaluated in non-tumor and tumor bearing (PC3 xenografted) male mice by *ex vivo* biodistribution studies and *in vivo* μPET imaging. Pretreatment studies were performed in the tumor bearing mice with the MET inhibitor PF04217903. Mouse plasma was analyzed with HPLC to quantify radiometabolites. To further evaluate the binding specificity of [^{18}F]cabozantinib, *in vitro* autoradiography studies on heart and PC3 tumor sections were performed in the presence of authentic cabozantinib or specific MET and VEGFR2 inhibitors.

Results: Tissue distribution studies in non-tumor bearing mice revealed slow blood clearance, absence of brain uptake and a high myocardial uptake. In the tumor bearing mice, tumor uptake was low ($0.58 \pm 0.20\%$ ID/g at 30 min post tracer injection), which was confirmed by μPET imaging. No differences in tissue distribution and kinetics were observed in both biodistributions and μPET studies after pretreatment with the MET inhibitor PF04217903. At 30 min post tracer injection, $60 \pm 3\%$ of the recovered radioactivity in plasma in non-tumor bearing mice was present as intact tracer. [^{18}F]cabozantinib binding *in vitro* to heart and tumor tissues was partly blocked in the presence of selective MET and VEGFR2 inhibitors (up to 40% block). The fraction of non-specific binding was relatively high for both tissues (66% for heart and 39% for tumor).

Conclusion: [^{18}F]cabozantinib exhibits non-favorable properties as a PET imaging probe, demonstrated by slow excretion kinetics along with low tumor uptake and high non-specific binding in tumor and heart tissue. The results reflect cabozantinib multi-kinase activity, making PET imaging of tumor specific kinase expression with [^{18}F]cabozantinib challenging.

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1. Introduction

Increased understanding of the molecular pathways that make cancer cells grow, survive and metastasize has led to more targeted treatments of cancer diseases [1]. An important class of such drugs is the tyrosine kinase inhibitors (TKIs) which prevent the phosphorylation of proteins important for the survival and proliferation of cancer cells [2].

Major research efforts in this area have led to more than 40 regulatory approved TKIs, with several others in clinical trials [3]. Despite these large developments, the response rates remain low [4]. Furthermore, due to rapid disease progression and severe side-effects associated with drug treatments, the correct kinase inhibitor regimen is imperative. However, patient stratification remains a challenge, and positron emission tomography (PET) can aid to identify responders from non-responders both in clinical trials and in clinical settings. Several TKIs have been labelled with positron emitters and evaluated as PET tracers in cancer models [5], exemplified with [^{18}F]afatinib [6] and [^{11}C]imatinib [7] in Fig. 1. By converting a known TKI to a positron-emitting

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Fig. 1. Chemical structures of [¹⁸F]afatinib and [¹¹C]imatinib, two regulatory approved TKIs already explored as oncological PET tracers. [¹⁸F]cabozantinib is evaluated herein.

isotopologue, valuable information about distribution, kinetics and target engagement in tumor tissue can be obtained *in vivo*. An advantage with isotopologues of already approved TKIs is that their toxicity has already been evaluated, which may facilitate the clinical translation of the PET tracer.

The tyrosine kinase MET is upregulated in many cancer types and has been associated with metastasis [8]. As most cancer deaths are related to metastasis, disease control is pivotal to increase life expectancy and quality [9]. To date, two TKIs inhibiting MET are regulatory approved: cabozantinib (Cabometyx/Cometriq, Exelixis) and crizotinib (Xalkori, Pfizer). Cabozantinib is a non-selective reversible TKI targeting MET, VEGFR2, KIT, FLT3 and AXL, inhibiting phosphorylation in cells with IC₅₀ values of 7.8, 1.9, 5.0, 7.5, and 42 μmol/L, respectively [10]. It is currently approved for the treatment of medullary thyroid cancer, renal cell carcinoma and hepatocellular carcinoma. Moreover, results from clinical trials have been reported for among others glioblastoma [11], lung [12] and breast cancer [13], and approximately 100 trials are currently ongoing [14]. Although prostate cancer trials with cabozantinib have not reached their endpoints [15,16], a reduction in metastatic bone lesions has been observed [17], indicating a clinical value of cabozantinib for this indication. Furthermore, the use of cabozantinib in combination with immunotherapy has been rationalized for prostate cancer [18], and clinical trials are ongoing. Since MET is known to correlate with poor prognosis and metastatic capability, it is of clinical interest to evaluate tumor expression of this kinase, and molecular imaging can be a valuable tool to accomplish this. To date, several PET studies aiming at imaging MET expression have been performed [19–24]. However, [¹¹C]SU11274 is the only tracer evaluated that binds intracellularly [25]. Since most small-molecule TKIs exert their effect through the intracellular ATP binding site, tracers that bind to the same active site can be important to properly assess target expression and drug engagement.

Due to the great interest in cabozantinib and the possibility of isotopologic change of the native fluorine in cabozantinib with [¹⁸F]

fluorine, [¹⁸F]cabozantinib has been reported in the patent literature and evaluated in a stereotactically implanted glioma model [26]. Here, a good tumor-to-background ratio was achieved; however, the nature of the applied xenograft model may yield an unnatural high tumor signal as the background in non-target brain tissue was very low. Following on our previous publication covering the synthesis of [¹⁸F]cabozantinib [27], the present study reports the *in vivo* evaluation of [¹⁸F]cabozantinib with focus on its biodistribution, kinetics and target engagement in male prostate cancer xenografted mice. The specificity of binding was further explored by *in vitro* autoradiography.

2. Material and methods

2.1. General

The authentic reference material cabozantinib and the boronic pinacol ester precursor were synthesized as described previously [27]. High-performance liquid chromatography (HPLC) for quality control and radiometabolite analysis was performed on a Shimadzu LC-2010A HT system connected to a UV spectrometer (254 nm) and a 3-inch NaI (TI) scintillation radiodetector with a single channel analyzer (Gabi box, Raytest, Straubenhardt, Germany). Radioactivity in samples of biodistribution studies and radiometabolite analysis was quantified with an automated gamma counter, equipped with a 3-inch NaI(Tl) well crystal coupled to a multichannel analyzer (Wallac 1480 Wizard, Wallac, Turku, Finland). The results were corrected for background radiation, detector dead-time and physical decay during counting. The MET inhibitor PF04217903 (methanesulfonate) and VEGFR2 inhibitor ZM323881 (hydrochloride) were purchased from Arkpharm Inc. (IL, USA) and Merck (Darmstadt, Germany), respectively. All animals were housed in individually ventilated cages in a thermoregulated (22 °C) and humidity-controlled environment under a 12 h/12 h day/night cycle with free access to food and water. Animal care and experiments were conducted in compliance with the Belgian Royal Decree of 29 MAY 2013, on the protection of laboratory animals. This Royal Decree

is in accordance with the European Council Directive No. 2010/63/EU of 01 JAN 2013 on the approximation of laws, regulations and administrative provisions regarding the protection of animals used for experimental and other scientific purposes. All animal experiments covering biodistribution, μ PET and plasma radiometabolite studies and section preparation for autoradiography were approved by the local Animal Ethics Committee of the University of Leuven (P176/2017). Animal care and experiments for xenograft generation were approved by the local Experimental Animal Ethics Committee of the BUC-CMMI (ref. CMMI-2013-05). The non-tumor bearing mice used in the *in vivo* studies were male naval medical research institute (NMRI) outbred mice purchased from Envigo ('HsdWin:NMRI'; Venray, The Netherlands). The tumor bearing mice were created from male NMRI nude mice purchased from Charles River labs ('CrI:NMRI-Foxn1^{nu}'; France).

2.2. Radiochemistry

[¹⁸F]cabozantinib was synthesized in a home-made remote-controlled synthesis module according to the previously described procedure [27] with some modifications. These modifications were implemented in order to adapt the procedure to another synthesis module (shift from ScanSys chemistry module (Værløse, Denmark) to a home-made remote-controlled module at KU Leuven), to improve the final chemical and radiochemical purity and to obtain a formulation suitable for intravenous injection. No-carrier-added [¹⁸F]fluoride (50–60 GBq) was produced via an ¹⁸O(p,n)¹⁸F nuclear reaction in a Cyclone 18/9 cyclotron (Ion Beam Applications, Louvain-la-Neuve, Belgium) by irradiation of 2 mL of 97% enriched [¹⁸O]H₂O (Rotem HYOX18, Rotem Industries, Beer Sheva, Israel) in a niobium target using 18-MeV protons. After irradiation for about 30 min, the resultant [¹⁸F]fluoride was separated from [¹⁸O]H₂O using a Chromafix® (PS-HCO₃) anion exchange cartridge (Machery-Nagel) that was converted to the C₂O₄²⁻ form by flushing with K₂C₂O₄ (0.5 M, 2.5 mL) followed by a rinse with water (Milli-Q, 2 × 2.5 mL) and dried under nitrogen flow. The [¹⁸F]fluoride was eluted from the cartridge into a 2-mL conical glass reaction vial using a solution of K₂C₂O₄ (1.9 mg) and Kryptofix 222 (7.4 mg) in H₂O/CH₃CN (0.2 mL, 5:95 v/v). The solution was evaporated under a stream of helium by applying conventional heating and further dried by azeotropic distillation with CH₃CN (2 × 1 mL) at a temperature of 110 °C. A blue solution of the boronic pinacol ester (10 mg, 0.016 mmol, [27]) and Cu(OTf)₂(py)₄ (15 mg, 0.022 mmol, Sigma-Aldrich) in anhydrous dimethylacetamide (400 μ L) was added to the dried [¹⁸F]fluoride/K₂C₂O₄/kryptofix complex, air was bubbled through the mixture for 10 s and the mixture was heated at 120 °C for 20 min. After cooling down, sodium phosphate buffer 10 mM pH 7.4 (1 mL) was added and the suspension was heated at 90 °C for 10 min to afford the hydrolysis of remaining precursor. CH₃CN (200 μ L) was added to improve the solubility and the milky suspension was filtered (empty 1 mL PP cartridge containing a 1 mm thick PE frit (Supelco, Sigma-Aldrich)) and loaded into the HPLC injection loop. Preparative HPLC purification was performed using an XBridge C18 OBD BEH column (5 μ m, 10 mm × 250 mm, Waters, Milford, USA) isocratically eluted with 45% EtOH in 10 mM Na₂HPO₄ at a flow rate of 3 mL/min. The column eluate was passed over a UV detector (254 nm) and a radiodetector and [¹⁸F]cabozantinib eluted at 21 min. To remove remaining free [¹⁸F]fluoride, a manual Sep-Pak (C18 Plus Light 130 mg, WAT023501, Waters) purification step was performed. The collected tracer fraction was diluted with water (15 mL) and loaded onto the C18 Sep-Pak cartridge (preconditioned with 5 mL EtOH and 10 mL water). After a rinse with 5 mL water, the tracer was eluted from the cartridge via a sterile filter (Millex-GV filter 0.22 μ m, 13 mm; Merck Millipore, MA, USA) into the final batch vial using EtOH (750 μ L) followed by dilution (via the SepPak and filter) with 0.9% NaCl solution (6.8 mL), resulting in a final batch formulation of 10% v/v ethanol in 0.9% w/v NaCl solution. Analytical HPLC analysis to confirm the identity and to determine the radiochemical and chemical purity and the molar activity was performed on an

XBridge C18 column (3.5 μ m, 3 mm × 100 mm, Waters) isocratically eluted with 40% CH₃CN in 10 mM Na₂HPO₄ at 0.6 mL/min. UV detection was set at 254 nm.

2.3. PC3 cell line culture

PC3 consists of human Prostate Cancer cells derived from bone metastasis of prostatic adenocarcinoma. 32-passages PC3 cells were grown in RPMI 1640 with L-Glutamine (ref. HYCLSH30027-02, GE Healthcare – Hyclone, United States) supplemented with 10% FBS (ref. 11550356, Gibco, Fisher Scientific, United States).

2.4. Xenograft generation

8 weeks old male nude NMRI mice were subcutaneously injected under gaseous anesthesia (induction: 3% isoflurane in O₂, 3 L/min; maintenance: 2% isoflurane in O₂, 1.5 L/min) with 5 × 10⁶ PC3 cells resuspended in 200 μ L of a mixture 1:1 RPMI 1640 serum-free (ref. HYCLSH30027-02, GE Healthcare – Hyclone, United States) and Matrigel (ref. 354248, Corning, Germany), with a 30 G insulin syringe (Eder et al. [28]). Mouse weight and tumor growth were monitored weekly during the whole experiment. Tumor measurements were performed using a digital caliper and tumor volume (TV) was calculated as TV = 0.5 (length × width [2]) in mm³. The tumors were allowed to grow for 3 to 4 weeks until approximately a volume of 1 cm³.

2.5. Biodistribution studies

Biodistribution of [¹⁸F]cabozantinib was studied in non-tumor bearing male mice (29–37 g, 6 weeks) and tumor bearing male mice (31–34 g, 11–12 weeks). The animals were anesthetized with isoflurane (2.5% in O₂, 1 L/min) and injected with 0.4–0.8 MBq of tracer (100–150 μ L; formulated in maximum 10% v/v EtOH in 0.9% w/v NaCl solution) via a tail vein. The non-tumor bearing mice were sacrificed by decapitation at 2, 10, 30 or 60 min post tracer injection (n = 3 per time point). The tumor bearing mice were either pretreated intraperitoneally (i.p.) at 30 min before tracer injection with the MET inhibitor PF04217903 (10 mg/kg, 300 μ L, n = 3) or left untreated (n = 3), and sacrificed by decapitation at 30 min p.i. The inhibitor was dissolved in an injectable solution composed of 15% v/v DMSO and 5% v/v Tween 80 in (2-hydroxypropyl)- β -cyclodextrin solution (Sigma-Aldrich, 40% w/v in water), and sterile filtered before administration (0.22 μ m membrane filter; Millex-GV, Millipore). Blood and organs of interest, in addition to the remainder of the animal, were collected in tared tubes, weighed and their radioactivity was counted in an automated gamma counter. The gall bladder was counted together with the liver. Data were expressed as percentage of injected dose per organ mass (% ID/g) and standardized uptake value (SUV). % ID/g was calculated as (counts per min (cpm) in organ / total cpm recovered) / organ mass × 100%. SUV was calculated as (cpm in organ / organ mass) / (total cpm recovered / total body mass). Data are expressed as mean $\pm$ SD.

2.6. Plasma radiometabolite analysis in male non-tumor bearing mouse

Non-tumor bearing male mice (n = 4) were anesthetized with isoflurane (2.5% in O₂, 1 L/min), injected i.v. with 11 MBq tracer (100–150 μ L; formulated in maximum 10% v/v EtOH in 0.9% w/v NaCl solution) via a tail vein, and sacrificed by decapitation at 30 min p.i. Blood was collected in EDTA-containing tubes (BD vacutainer, BD, Franklin Lakes, NJ, USA) and stored on ice. The blood was centrifuged (10 min, 4000 rpm) to separate the plasma and an equal volume of CH₃CN was added to precipitate plasma proteins. After centrifugation (10 min, 4000 rpm), the supernatant was filtered and spiked with the authentic reference cabozantinib. Plasma was then analyzed with RP-HPLC on an XBridge C18 column (3.5 μ m, 3 mm × 100 mm, Waters) eluted with 40% CH₃CN

in 10 mM Na₂HPO₄ buffer at 0.6 mL/min. HPLC eluent was collected in 1 mL fractions and radioactivity in all fractions was measured in an automated gamma counter.

2.7. μ PET imaging of tumor bearing mice

Whole-body PET scans were acquired on a FOCUS™ 220 μ PET scanner (Concorde Microsystems, Knoxville, USA). Tumor bearing mice ($n = 6$) were anesthetized with isoflurane (2.5% in O₂, 1 L/min) and 11 MBq of [¹⁸F]cabozantinib (100–150 μ L; formulated in maximum 10% v/v EtOH in 0.9% w/v NaCl solution) was injected *via* a tail vein. Dynamic scans were acquired for 120 min, and the mice were kept under anesthesia (2.5% isoflurane in O₂, 1 L/min) for the entire scan period. Of the six mice that were scanned baseline, three received an additional pretreatment with PF04217903 (10 mg/kg, 400 μ L, prepared as described above) i.p. at 30 min before tracer injection. After one week, the three mice were again anesthetized using isoflurane (2.5% in O₂, 1 L/min) and injected i.v. with 11 MBq 2-[¹⁸F]fluoro-2-deoxy-D-glucose ([¹⁸F]FDG). The mice were allowed to recover consciousness during the uptake period of [¹⁸F]FDG. At 60 min post tracer injection, the mice were anesthetized with isoflurane (2.5% in O₂, 1 L/min) and submitted to a 10 min static PET scan. Acquisition data of the dynamic scans were Fourier rebinned in 24 time frames (4 $\times$ 15 s, 4 $\times$ 60 s, 5 $\times$ 180 s, 8 $\times$ 300 s, 3 $\times$ 600 s). Time activity curves (TACs) of the tumors were generated using PMOD software (v3.3, PMOD Technologies, Zürich, Switzerland). All mice recovered from the scans.

2.8. *In vitro* autoradiography

[¹⁸F]cabozantinib (molar activity of 10–20 GBq/ μ mol) was diluted to the appropriate radioactive concentrations (5–20 kBq/mL) with PBS buffer (pH 7.4, 10 mM). Frozen heart and PC3 tumor tissue sections (20 μ m) were thawed, dried and preincubated in PBS buffer (pH 7.4, 10 mM) for 10 min at room temperature. After drying, the sections were incubated at room temperature for 30 min with 200 μ L (1–4 kBq) of [¹⁸F]cabozantinib solution containing either 10 μ M of a blocking agent (authentic cabozantinib, PF04217903 or ZM323881) or an equivalent volume of DMSO ($n = 6$ for each condition and tissue). Sections were washed for 2 $\times$ 5 min in PBS (pH 7.4, 10 mM), followed by a short rinse in ice-cold water. All PBS solutions used in the incubation and washing steps contained 0.3% bovine serum albumin (BSA). After drying, the sections were exposed to a phosphor storage screen (Fujifilm SR High Res, Fujifilm, Japan) overnight. The screens were read with a Fujifilm BAS-5000 plate reader. The results were extracted as PSL/mm² (photostimulated luminescence per square millimeter) and compared using the *t*-test. The effect of the various blocking agents in percent were calculated with the formula $100 \times (\text{PSL/mm}^2_{\text{control}} - \text{PSL/mm}^2_{\text{block}}) / \text{PSL/mm}^2_{\text{control}}$ using averaged PSL/mm² values.

3. Results

3.1. Radiochemistry

Radiosynthesis of [¹⁸F]cabozantinib was performed as previously reported using copper-mediated chemistry starting from the corresponding boronic ester precursor (Scheme 1) [27]. However, some modifications were made to implement the production in another synthesis platform: A filtration prior to HPLC purification was included to remove undissolved material thereby preventing clogging of the injector or column. C18 Sep-Pak reformulation after preparative HPLC purification removed residual unreacted [¹⁸F]fluoride and increased the radiochemical purity. Furthermore, the original gradient protocol for preparative HPLC was replaced by an isocratic protocol. Representative chromatograms of an HPLC purification and a quality control HPLC analysis can be found in the Supporting information (Figs. S1, S2, S3). Using this procedure, [¹⁸F]cabozantinib was produced in a total synthesis time of 1 h 45 min with an average decay-corrected (EOB) radiochemical yield of $4.2 \pm 0.6\%$, an average molar activity of 187 ± 38 GBq/ μ mol and a radiochemical purity of $> 99\%$ ($n = 5$, total batch radioactivity 1.1–1.5 GBq).

3.2. Biodistribution

In the non-tumor bearing mice, a high uptake was observed in both liver and kidneys (Fig. 2a and Supporting information Table S1), and radioactivity in blood was slowly decreasing from 2.20 ± 0.20 to $1.71 \pm 0.55\%$ ID/g from 2 to 60 min post tracer injection. At 60 min after tracer injection, $11.72 \pm 1.30\%$ ID/g was present in the liver, whereas kidney uptake was lower ($7.71 \pm 1.42\%$ ID/g). Uptake in the heart was relatively high ($15.96 \pm 1.48\%$ ID/g at 2 min p.i. and $4.86 \pm 1.34\%$ ID/g at 60 min p.i.), while bone and brain uptake were negligible. The overall distribution in non-tumor and tumor bearing animals after 30 min was comparable, and a low tumor uptake ($0.58 \pm 0.20\%$ ID/g) was observed (Fig. 2b). Radioactivity concentration in blood ($1.16 \pm 0.10\%$ ID/g) and muscle ($3.37 \pm 0.27\%$ ID/g) were higher than in tumor tissue, yielding low tumor-to-blood ratio of 0.5 and tumor-to-muscle ratio of 0.17. For the pretreated tumor bearing mice, a similar tumor uptake of $0.55 \pm 0.23\%$ ID/g was observed, and no difference in overall tissue distribution was observed after i.p. pretreatment with PF04217903 (10 mg/kg, 30 min prior to tracer injection, 300 μ L). Additional biodistribution data can be found in the Supporting information (Table S2 and Figs. S4 and S5).

3.3. μ PET imaging of tumor bearing mice

Despite the low tumor uptake observed in the biodistribution studies, the tumors were visible in the μ PET baseline images (Fig. 3a, left). Tumor location was confirmed with an [¹⁸F]FDG scan (Fig. 3a, right). Imaging of animals pretreated with PF04217903 showed no difference in

Scheme 1. Synthesis of [¹⁸F]cabozantinib from the corresponding boronic ester precursor using copper-mediated chemistry.

Fig. 2. a) Biodistribution of $[^{18}\text{F}]$ cabozantinib at $t = 2, 10, 30$ and 60 min ($n = 3$ at each time point) in non-tumor bearing mice. b) Biodistribution of $[^{18}\text{F}]$ cabozantinib at 30 min post tracer injection in tumor bearing mice. Values are compared between baseline ($n = 3$) and pretreatment ($n = 3$) with PF04217903. Animals were anesthetized with 2.5% isoflurane in O_2 at a flow rate of 1 L/min. PF04217903 (10 mg/kg) was administered i.p. 30 min before i.v. tracer injection (0.4 – 0.8 MBq).

tumor uptake compared to untreated animals (images not shown). Time activity curves (TACs) were extracted, showing that PF04217903 was not able to displace tracer binding in the tumor tissue (Fig. 3b).

3.4. Plasma radiometabolite analysis in male non-tumor bearing mice

The *in vivo* metabolic stability of $[^{18}\text{F}]$ cabozantinib in male mouse plasma was analyzed at 30 min post tracer injection using HPLC. A reconstructed radiochromatogram can be found in the Supporting information (Fig. S6). $60 \pm 3\%$ ($n = 4$) of the recovered radioactivity was present as intact tracer, and only more polar radiometabolite(s) were observed.

3.5. *In vitro* autoradiography

The binding specificity of $[^{18}\text{F}]$ cabozantinib to PC3 tumor and heart tissue was evaluated by coinubation of the tracer with cabozantinib, the selective MET inhibitor (PF04217903) [29] or the selective VEGFR2

inhibitor (ZM323881) [30]. In baseline conditions, a twofold higher binding intensity was observed for heart compared to tumor tissue. Both specific blocking agents were able to partially inhibit tissue binding of $[^{18}\text{F}]$ cabozantinib, but to a lesser degree than cabozantinib (Fig. 4). For tumor tissue, cabozantinib, PF04217903 and ZM323881 were able to block tracer binding by 61%, 25% and 40%, respectively, while for the heart these values were 34%, 27% and 26%. For all conditions, except between heart blocked with PF04217903 or ZM323881, the differences were statistically significant ($p < 0.0001$).

4. Discussion

$[^{18}\text{F}]$ cabozantinib was obtained with copper-mediated fluorination from a boronic pinacol ester precursor as previously reported [27]. Using that protocol, small amounts of hydrolyzed precursor remained present after HPLC purification of $[^{18}\text{F}]$ cabozantinib, reducing the chemical purity. In the synthesis reported herein, a filtration step was included before the HPLC purification, a switch was made from a

Fig. 3. μ PET imaging of tumor bearing mice. a) Averaged summed baseline image of a 120 min dynamic scan after injection of 11 MBq $[^{18}\text{F}]$ cabozantinib, and μ PET image derived from a 10 min static scan (60 min p.i.) after i.p. injection of 11 MBq $[^{18}\text{F}]$ FDG. The location of the tumor is indicated by a red arrow. b) Corresponding time activity curves (TACs) for the tumor region for baseline ($n = 6$) and pretreated ($n = 3$) animals. Animals were anesthetized with 2.5% isoflurane in O_2 at a flow rate of 1 L/min and kept under anesthesia during the entire scan period (120 min). PF04217903 (10 mg/kg) was administered i.p. 30 min before i.v. tracer injection (11 MBq).

Fig. 4. In vitro autoradiography binding study of [^{18}F]cabozantinib (4 kBq) on PC3 tumor and heart tissue sections, performed in the presence of different blocking agents (10 μM). All groups were significantly different from each other, except heart blocked with PF04217903 or ZM323881.

gradient to an isocratic preparative HPLC method and a Sep-Pak reformulation was included. As a result, the traces of hydrolyzed precursor were efficiently removed and the amount of free [^{18}F]fluoride was reduced, increasing the chemical- and radiochemical purity of the final [^{18}F]cabozantinib batch. A chemical impurity derived from protodeboronation of the precursor was closely eluting with [^{18}F]cabozantinib and proved difficult to remove entirely. This impurity was present in only low amounts (10–20% of cold mass) and with the observed high molar activity of the final formulated [^{18}F]cabozantinib, the impurity is likely to not confound the results in the *in vivo* evaluation. The successful transfer of the radiochemistry between different laboratories shows that the copper-mediated radiosynthesis of [^{18}F]cabozantinib is robust and adaptable to various semi-automated synthesis modules.

[^{18}F]cabozantinib was evaluated *in vivo* for its potential as a tumor imaging agent. *Ex vivo* biodistribution in non-tumor bearing mice indicated predominantly liver excretion, as was expected given the lipophilicity of the tracer ($\log D_{7.4} = 5.15$) [31] and earlier reported pharmacokinetic evaluation of cold cabozantinib in human and of [^{14}C]cabozantinib in rat and dog [32]. In addition, relatively high blood activity and slow washout were observed which can be explained in terms of high plasma protein binding (99%) [32]. Tracer concentration in heart tissue was significantly higher than in blood at all studied time points, indicating that the heart uptake is not solely due to a well-perfused heart. The heart uptake observed with [^{18}F]cabozantinib is notably higher than what has been observed with other TKI PET tracers [6,33,34].

Although cabozantinib has shown treatment response in glioblastoma multiforme [11] and brain metastatic renal cancer [35], [^{18}F]cabozantinib showed negligible brain uptake in this study. Only a limited number of TKIs are able to cross the blood-brain barrier (BBB), and many are substrates of efflux pumps [36]. Across a range of drug transporter enzymes, cabozantinib is only reported to be a substrate of multidrug resistance-associated protein 2 (MRP2) [32], leaving room for the possibility of CNS accumulation. The observed therapeutic effect in CNS cancers may be due to a partly disrupted BBB [37] and higher drug concentrations at therapeutic doses.

To evaluate the tumor uptake of [^{18}F]cabozantinib, biodistribution and μPET imaging studies were performed in tumor bearing (PC3 xenografted) mice. The PC3 tumor cell line was chosen based on the reported overexpression of MET [38–40] and the effect of cabozantinib on tumor xenografts in mice [41,42]. Despite a relatively low uptake ($0.58 \pm 0.20\%$ ID/g at 30 min) observed in the *ex vivo* biodistribution, the tumor could be localized in the μPET images (Fig. 3a). The low tumor uptake combined with the slow washout from blood for [^{18}F]cabozantinib reflects its characteristics as a therapeutic molecule. High lipophilicity and plasma protein binding are favorable for a prolonged pharmacological activity but will result in a low tumor accumulation

in the shorter time frame applied in PET studies. In general, since TKIs exert their effects intracellularly, difficulties for TKI PET tracers to reach the target may be present. In addition, since PET utilizes a very low dose compared to the therapeutic doses, the tracer may be displaced by high concentrations of intracellular ATP competing for the same site. The synthesis and evaluation of [^{18}F]cabozantinib described in a patent application applied a stereotactically implanted glioma model, and more favorable tumor-to-background ratios were observed [26].

The observed tumor uptake did not decrease after pretreatment with the MET inhibitor PF04217903, indicating that tumor binding of [^{18}F]cabozantinib is not mediated by MET. Comparison of the TACs for the baseline and pretreated tumor bearing mice confirmed the results of the *ex vivo* biodistribution, with no significant blocking effect after PF04217903 pretreatment (Fig. 3b). A possible explanation is the multi-kinase activity of cabozantinib combined with low target expression and high background signal, making a distinction of *in vivo* binding of [^{18}F]cabozantinib to MET or other kinases impossible. To get more insight into the binding specificity of [^{18}F]cabozantinib, *in vitro* autoradiography on PC3 tumor sections was performed. Due to the high tracer uptake in heart tissue observed *in vivo*, *in vitro* autoradiography was also performed on heart sections. Specific binding was assessed by coincubation with either cabozantinib, a selective MET inhibitor (PF04217903) [29] or a selective VEGFR2 inhibitor (ZM323881) [30], each at a concentration of 10 μM . In both tumor and heart tissue, non-specific binding was relatively high and more pronounced in heart compared to tumor tissue (66% for heart and 39% for tumor). Neither the MET nor the VEGFR2 selective blocker was able to displace all specific binding, which substantiates the multi-kinase nature of cabozantinib. Cardiotoxicity of cabozantinib has been observed [43,44], which may be related to the presence of specific binding of cabozantinib to heart tissue.

At 30 min post injection, $60 \pm 3\%$ of the radioactivity in male mouse plasma corresponded to the intact tracer, and the observed radiometabolite(s) were more polar compared to [^{18}F]cabozantinib. Their identity was not determined, but demethylation, cleavage of amide bonds and *N*-oxidation processes can be expected, as these are the main metabolites observed in previous studies of cabozantinib. All of these are more hydrophilic, and exhibit lower affinities for the various kinases known to be targeted by cabozantinib [32]. Cabozantinib is mainly metabolized by CYP3A, which is sex-specific, and metabolite studies should therefore be treated cautiously, also with regards to interspecies differences. No increase in bone uptake was observed during the time frame of the *in vivo* evaluation, reflecting the stability of the aryl-fluoride bond.

5. Conclusion

In vivo evaluation of [^{18}F]cabozantinib as a PET imaging agent in PC3 tumor bearing mice revealed slow excretion kinetics with high background in non-target organs, and low tumor uptake which could not be blocked with a selective MET inhibitor. Combined with *in vitro* autoradiography data, this suggests that [^{18}F]cabozantinib lacks the properties of a useful PET imaging probe.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nucmedbio.2020.12.002>.

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