 Development and evaluation of molecular imaging probes for CXCR4 mediated chemotaxis and tumor infiltration of activated T-Cells
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Document Version
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Publication date: 2015

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 3:


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Mol Pharm. 2014; 11(11):3810-17
Chapter-3

ABSTRACT

The chemokine receptor CXCR4 and its ligand CXCL12 play an important role in tumor progression and metastasis. CXCR4 receptors are expressed by many cancer types and provide a potential target for treatment. Non-invasive detection of CXCR4 may aid diagnosis and improve therapy selection. It has been demonstrated in preclinical studies that position emission tomography (PET) with a radiolabelled small molecule could enable noninvasive monitoring of CXCR4 expression. Here, we prepared N-[\(^{11}\)C]methyl-AMD3465 as a new PET tracer for CXCR4. N-[\(^{11}\)C]methyl-AMD3465 was readily prepared by N-methylation with [\(^{11}\)C]CH\(_3\)OTf. The tracer was obtained in a 60±2% yield (decay corrected), the purity of the tracer was >99% and specific activity was 47±14 GBq/µmol. Tracer stability was tested in-vitro using liver microsomes and rat plasma; excellent stability was observed. The tracer was evaluated in rat C6 glioma and human PC-3 cell lines. In vitro cellular uptake of N-[\(^{11}\)C]methyl-AMD3465 was receptor mediated. The effect of transition metal ions (Cu\(^{2+}\), Ni\(^{2+}\) and Zn\(^{2+}\)) on cellular binding was examined in C6 cells and the presence of these ions increased the cellular binding of the tracer 9, 7 and 3 fold, respectively. Ex-vivo biodistribution and PET imaging of N-[\(^{11}\)C]methyl-AMD3465 were performed in rats with C6 tumor xenografts. Both PET and biodistribution studies demonstrated specific accumulation of the tracer in the tumor (SUV 0.6±0.2) and other CXCR4 expressing organs, such as lymph node (1.5±0.2), liver (8.9±1.0), bone marrow (1.0±0.3), and spleen (1.0±0.1). Tumor uptake was significantly reduced (66%, \(p<0.01\)) after pre-treatment with Plerixafor (AMD3100). Biodistribution data indicates that a tumor-to muscle ratio of 7.85 and tumor- to plasma ratio of 1.4, at 60 min after tracer injection. Our data demonstrated that N-[\(^{11}\)C]methyl-AMD3465 is capable of detecting physiologic CXCR4 expression in tumors and other CXCR4 expressing tissues. These results warrant further evaluation of N-[\(^{11}\)C]methyl-AMD3465 as a potential PET tracer for CXCR4 receptor imaging.
INTRODUCTION

The chemokine receptor 4 (CXCR4) is overexpressed in more than 20 different human cancers [1, 2] and was shown to be an independent prognostic factor in several types of cancer [3]. CXCR4 and its ligand stromal cell derived factor 1α (CXCL12) play a pivotal role in tumor cell homing and metastasis. The organs with highest CXCL12 expression represent the first metastatic locations of CXCR4 expressing tumors. This phenomenon can be ascribed to CXCL12-induced chemotaxis, which stimulates the migration of CXCR4 receptor expressing tumor cells towards its concentration gradient [4]. Inhibition of the CXCR4 mediated chemotaxis with e.g. monoclonal antibodies or CXCR4 antagonists could significantly reduce the metastatic potential of the tumor and improve survival [5,6]. CXCR4 antagonists can also be used to sensitize tumor cells, as these antagonists can disrupt CXCR4-dependent tumor-stromal interactions. In several cancer models, better response was achieved when conventional chemotherapy and/or radiotherapy was combined with a CXCR4 inhibitor, such as AMD3100 (Plerixafor) [7-9]. Considering the above, early detection of CXCR4 receptor expression in the tumor may be useful as a prognostic factor or biomarker to select patients eligible for treatment with CXCR4 antagonists, provided that a suitable diagnostic tool is available. Our goal is to develop a diagnostic imaging agent that allows detection of CXCR4 expression with high specificity and sensitivity. Recently, we reported $^{99m}$TcO$_2$-AMD3100 as a probe for SPECT imaging of CXCR4 receptors in a PC-3 tumor xenograft mouse model. The results of this study were modest, because incorporation of technetium-99m in the cyclam ring of the CXCR4 antagonist AMD3100 resulted in substantially reduced binding affinity towards the receptor. As a result, uptake of the tracer in the tumor was relatively low (0.4 %ID/g, after 60 min) [10]. Several other scintigraphic imaging probes for CXCR4 receptors, including proteins, peptides and small molecules have been developed over the past decades [10-19]. While some of these compounds showed potential in preclinical evaluations, none of these agents is used in clinical practice so far. AMD3465 is a small molecule CXCR4 antagonist that is more selective and has higher binding affinity than the anti-CXCR4 drug Plerixafor (AMD3100) [20-23].
AMD3465 consists of a monocyclam ring connected via a linker with an aminomethyl-pyridine moiety and is a structural analogue of AMD3100. The secondary amino group in the linker allows the introduction of a small substituent and therefore is available for labeling of the antagonist with carbon-11. In this study, we developed N-[\(^{11}\)C]methyl-AMD3465 as a new PET tracer for imaging of CXCR4 receptors and evaluated its biological properties in a tumor model.

**MATERIALS AND METHODS**

**Materials and Instruments**

All reagents were obtained from commercial suppliers and used without further purification. Human liver microsomes were obtained from BD biosciences. HPLC analyses were performed on a Waters system, consisting of a 515-isocratic pump, a multi-wavelength UV detector operated at 220 nm and a Bicron Geiger–Müller radioactivity detector. A description of the synthesis of the labeling precursor and the N-methyl-AMD3465 reference standard is provided in the Supporting Information (Supplement Figure-1).

**Labeling of N-[\(^{11}\)C]methyl-AMD3465**

\([^{11}\)C]CH\(_3\)I was prepared as was previously described [25]. \([^{11}\)C]Methyl triflate was formed by passing the \([^{11}\)C]methyl iodide through a column of silver triflate bound on \(\alpha\)-alumina at 240°C with a flow of 25 mL/min. \([^{11}\)C]Methyl triflate was transferred by a stream of helium (25 mL/min) into a reaction vial containing 0.4 mg of N,N',N''-tri(trifluoroacetyl)-AMD3465 in 300 \(\mu\)L dry acetone, cooled in an ice bath. After trapping of \([^{11}\)C]methyl triflate, the reaction mixture was heated at 80°C for 5 min. Subsequently, 300 \(\mu\)L of methanol and 100 \(\mu\)L of NaOH (1M) were added and the reaction mixture was heated at 80°C for another 5 min. The reaction mixture was neutralized with 70% phosphoric acid (16 \(\mu\)L), diluted with 0.6 mL HPLC eluent and purified by HPLC using a Zorbax SB C18 column (250 x 7.8 mm) and sodium phosphate buffer (100 mM, pH 2.0)/EtOH (95/5 v/v) as the eluent at a flow of 4 mL/min. The radioactive product with a retention time of 12±1 min was collected (10-12 mL), neutralized with 1M NaOH (400 \(\mu\)L, pH ~7.0) and passed over a Millex 0.22-\(\mu\)m GV filter (Millipore, Ireland) to yield a sterile solution of
N-[¹¹C]methyl-AMD3465 ready for injection. An aliquot of the product solution was taken for quality control by HPLC using a Jupiter C18 column (300 x 7.8 mm, Phenomenex) and water (adjusted to pH 2.0 with HClO₄/acetonitrile (90/10) as the eluent at a flow of 1 mL/min (retention time: 8±1 min).

**Distribution Coefficient**

The distribution coefficient (LogD) of N-[¹¹C]methyl-AMD3465 was determined by adding 5 MBq of the tracer to a mixture of 5 mL n-octanol and 5 mL of phosphate buffer saline (PBS). The mixture was vortexed, and incubated at 37°C for 1 h. After incubation, the mixture was centrifuged at 6000 rpm for 2 min. 100 μL aliquots were collected from octanol and aqueous layers and radioactivity was measured (Compugamma CS1282, LKB-Wallac, Turku, Finland). The LogD value is reported as the average of five independent experiments.

**In vitro stability towards human liver microsomes**

To 50 µL of 0.5 mg/mL liver microsome solution in Dulbecco’s PBS (DPBS, pH 7.4) and 2 µL of 4 mM N-methyl-AMD3465 in DPBS, 50 µL of 1 mM NADPH in DPBS and 400 µL DPBS were added. The mixture was shaken in a water bath at 37°C. Aliquots of 100 µL were taken at 0, 5, 15, 30, 45, 60, 90 and 120 min. An identical mixture without NADPH was used as a control. Each aliquot was immediately quenched in a solution of 0.1% formic acid in 500 µL acetonitrile. The samples were vortexed and centrifuged at 6000 rpm for 5 min. An aliquot of 400 µL was transferred into an ice-cold vial for LC-MS analysis. A verapamil solution with a known concentration (2mM) was used as an internal standard.

**Stability in rat plasma**

About ~150 MBq of N-[¹¹C]methyl-AMD3465 was dissolved in 2 mL of freshly isolated rat plasma and incubated at 37°C for 2 h. After 5, 15, 30, 45, 60, 90, and 120 min, an aliquot of 400 µL was taken and 25 µL 70% perchloric acid was added. The sample was centrifuged at 6000 rpm for 5 min. The supernatant was injected into an HPLC system equipped with a μBondapak column (300 x 7.8 mm). The sample was eluted with water (adjusted to pH 2.0 with HClO₄/acetonitrile (90/10)
as the eluent at a flow of 3 mL/min. The eluate was collected in 30 s fractions and radioactivity was measured. All the experiments were repeated in triplicate.

**Cell cultures**

C6 rat glioma cells (ATCC, Manassas, VA) were cultured in monolayers in DMEM supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine. PC-3 human prostate cells were grown in RPMI supplemented with 10% FCS. Cells were maintained in a humidified atmosphere with 5% CO$_2$ at 37°C.

**Receptor Binding assays**

Cells were grown in monolayers in 12-well plates. After the cells were washed with 2 mL PBS, 1 mL binding buffer, consisting of PBS with 5 mM MgCl$_2$, 1 mM CaCl$_2$, and 0.25% BSA was added. After 5 MBq N-[${}^{11}$C]methyl-AMD3465 was added, cells were incubated at 37°C. At different time points, the medium was removed and the cells were washed twice with 1 mL cold PBS. Cells were trypsinized (200 µL) and collected in 1 mL medium. The cell bound activity was measured. A 50 µL sample of the suspension was mixed with 50 µL of trypan blue and used for cell counting. Cell numbers were manually counted, using a phase-contrast microscope (Zeiss), a Burker bright-line chamber, and a hand-tally counter. In competition binding studies, various concentrations (range $10^{-8}$ to $10^{-4}$ M) of the CXCR4 antagonists AMD3100, AMD3465, or N-methyl-AMD3465 were added to the medium before the cells were incubated with the tracer at 37°C for 30 min. To test the effect of transition metals on tracer binding, 2.5 µM of CuSO$_4$, ZnCl$_2$, NiCl$_2$, NaRhO$_4$ or Pd(OAc)$_2$ was added to the medium immediately before tracer incubation. To determine the ED$_{50}$ of CuSO$_4$ and NiCl$_2$, cells were incubated with different concentrations of these transition metals in the range of $10^{-7}$ to $10^{-3}$ M. The IC$_{50}$ and EC$_{50}$ values were determined by nonlinear regression analysis, using GraphPad prism 5.0. All experiments were performed in triplicate.

For internalization assays, first C6 cells were incubated with 5-10 MBq of N-[${}^{11}$C]methyl-AMD3465 for 30 min at 4°C, assuming very low level of internalization or receptor-mediated endocytosis at this temperature. The medium with unbound radioactivity was removed and cells were washed twice with ice-cold
PBS. Then, cells were incubate with fresh binding buffer at 37°C for 0, 15, 30, 45, or 60 min. Free and extracellular bound activity was removed by washing with 100 μl 50 mM glycine-HCl in 10 mM NaCl (pH 2.8) and cells were washed twice with ice cold-PBS. These wash fractions were added to the glycine.HCl fraction (surface bound fraction). Subsequently, cells were lysed with 200 μL 1M NaOH (internalized fraction). Radioactivity in both lysate and acid wash fractions were measured. The percentage of internalization was calculated as:

Internalization (%) = \[
\frac{\text{Internalized activity}}{\text{surface-bound activity} + \text{Internalized activity}} \times 100
\]

**Animal Model**

The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Groningen (DEC6073D). Male Wistar rats (280-350g, Harlan) were allowed 1 week of acclimation after arrival. The rats were maintained at 12 h day/night regime and fed standard laboratory chow. Three million C6 glioma cells in a 1:1 (v/v) mixture of matrigel and complete medium were subcutaneously injected into the right shoulder. Solid tumors (0.4 ± 0.2 g) were allowed to grow for 5-6 days before the animals were scanned.

**Small-Animal PET and ex-vivo biodistribution**

Animals were anesthetized with a mixture of isoflurane/air (5% for induction, 2% for maintenance). The rats were positioned in the PET camera (Focus 220, Siemens-Concorde) with the tumor in the field of view. A 15 min transmission scan was made for attenuation and scatter correction. Then, 20±5 MBq of N-[11C]methyl-AMD3465 was injected via the penile vein and a 60-min dynamic emission scan of the tumor region was started. Animals were terminated and relevant tissues were excised and weighed. Radioactivity in these tissues was measured. Tracer uptake was expressed as standardized uptake value (SUV). In blocking studies, the CXCR4 receptors were saturated by subcutaneous injection of 30 mg/kg AMD3100.8HCl 30 min before tracer injection. The list mode data of the emission scan were separated into 21 frames (6x10, 4x30, 2x60, 1x120, 1x180, 4x300, 3x600s). Emission sinograms were iteratively reconstructed (OSEM2D, 16 subset, 4 iterations) after
being normalized and corrected for attenuation, scatter and radioactive decay. Volumes of interest (VOIs) were manually drawn on the summed PET images and time activity curves were calculated, using standard software (Inveon Research Workplace, Siemens-Concorde). Time-activity curves are presented as SUV.

**Immunohistochemistry**

Tumor immunostaining was performed as described in the literature [10]. In brief, frozen tumor sections were cut into 5-µm-thickness and fixed in acetone followed by immersing in a PBS solution containing 0.5% hydrogen peroxidase for 30 min to block endogenous peroxidase activity. To reduce the non-specific binding, the sections were incubated with 2.5% goat serum for 30 min at room temperature. Then, sections were incubated overnight with primary rabbit polyclonal anti-CXCR4 antibody (Abcam, clone2074; Cambridge, UK, at 1: 500 dilution) at 4°C. Subsequently, sections were processed using a standard horseradish peroxidase (HRP) conjugated secondary and tertiary antibody according to the manufacturer’s recommendations (Dako, Belgium). Diaminobenzidine (DAB) was used as a chromogen, and hematoxylin was used for counterstaining. After washing, cover slips were mounted and sections were examined under a Leica microscope. A negative control without primary antibody was used to check the specificity of the antibody.

**Statistical Analysis**

Statistical analyses were performed using the two-sided unpaired students’ t-test in GraphPad prism 5 or Sigma plot (2001). Probability (p) values less than 0.05 were considered statistically significant.

**RESULTS**

**Radiochemistry**

N-[¹¹C]methyl-AMD3465 was reliably prepared by methylation of the trifluoroacetyl protected precursor (Fig-1). After HPLC purification, the tracer was obtained in 60±2% yield (based on [¹¹C]CH₃OTf, corrected for decay). The tracer was >99% pure with a specific activity of 47±14 GBq/µmol. The total synthesis time was approximately 50 min. The LogD of the tracer is -0.86±0.09.
**Figure-1:** Synthesis of N-[\(^{11}\)C]methyl-AMD3465. Reagents and conditions: (i) [\(^{11}\)C]CH\(_3\)OTf, Acetone, 80\(^\circ\)C, 5 min (ii) MeOH, 1M NaOH, 5 min.

**Stability and cellular accumulation of N-[\(^{11}\)C]methyl-AMD3465**

N-[\(^{11}\)C]methyl-AMD3465 was highly stable towards both human liver microsomes and rat plasma. In both cases, >99 % of the tracer was still intact after 2 h of incubation (Fig-2A and 2B). The *in-vitro* binding of N-[\(^{11}\)C]methyl-AMD3465 to C6 and PC-3 cells reached a maximum within 30 min (Fig-3A). Cellular uptake was slightly higher in C6 than PC-3 cells, but this difference was not statistically significant. Cellular accumulation of N-[\(^{11}\)C]methyl-AMD3465 was CXCR4 receptor mediated, as tracer binding could be saturated by CXCR4 antagonists (Fig-3B and 3C). The IC\(_{50}\) values of the CXCR4 antagonists AMD3465, N-methyl-AMD3465 and AMD3100 in PC-3 cells were 0.68±0.01 µM, 1.1±0.03 µM and 3.0±0.02 µM, respectively. In C6 cells, the IC\(_{50}\) values were 0.20±0.05 µM, 0.45±0.09 µM and 1.5±0.07 µM, respectively.

**Figure-2:** Stability test (n=3). A) Stability tests of N-methyl-AMD3465 in human liver microsomes don’t show any detectable metabolites. B) *In-vitro* stability analysis of N-[\(^{11}\)C]methyl-AMD3465 in rat plasma indicates that the tracer is stable up to at least 2 h.

Approximately 40% of the tracer was internalized within 15 min and reached a plateau there after (Fig-3D). The presence of certain transition metal ions significantly (\(p<0.005\)) increased the cellular binding of N-[\(^{11}\)C]methyl-AMD3465 in C6 cells, in
particular copper (9 fold), nickel (7 fold) and zinc ions (~3 fold). In contrast, in the presence of rhenium and palladium, the binding of the tracer was slightly reduced (Fig-4A). Copper and nickel affected cellular uptake of N-[11C]methyl-AMD3465 at a similar concentration, with EC50 values of 55 µM and 59 µM, respectively (Fig-4B and 4C).

Figure-3: In-vitro characterization of N-[11C]methyl-AMD3465 (all experiments were performed in triplicate): A) Cellular uptake kinetics in C6 and PC-3 cells. B) CXCR4 receptor binding of N-[11C]methyl-AMD3465 in C6-glioma cells in the presence of CXCR4 antagonists. C) CXCR4 receptor binding of N-[11C]methyl-AMD3465 in human PC-3 prostate cancer cells in the presence of CXCR4 antagonists. Cells were incubated with the tracer at 37°C for 30 min. D) Internalization of N-[11C]methyl-AMD3465 in C6 cells.

Ex vivo biodistribution

N-[11C-]methyl-AMD3465 mainly accumulated in excretory organs and in organs with high CXCR4 expression (Fig-5). Sixty minutes after tracer injection, the highest accumulation was found in kidney (SUV 12±1.0) and liver (SUV 8.9±1.0); followed by lymph node (1.5±0.2), bone marrow (1.0±0.3), spleen (1.0±0.1), thymus (0.5±0.1), adrenal glands (1.0±0.2) and tumor (0.6±0.2). Sixty min after tracer
injection, the tumor-to-muscle ratio was 7.8±1.3 and the tumor-to-blood ratio was 1.42±0.23. Pre-treatment with the selective CXCR4 antagonist AMD3100.8HCl (30 mg/kg) resulted in a significant reduction in tracer uptake in thymus (36%), adrenal gland (47%), spleen (52%), bone marrow (72%), tumor (66%), liver (30%) and lymph node (86%).

Figure-4: Effect of transition metals on the cellular uptake of N-[11C]methyl-AMD3465 in C6 cells. A) Cellular uptake in the presence of different transition metal ions after incubation at 37°C for 30 min. B) and C) The concentration dependent effect of copper and nickel ions on the cellular uptake of N-[11C]methyl-AMD3465 in C6 cells respectively. Significant differences are indicated with **(p<0.01) and ***(p<0.005). All experiments were performed in triplicate.

Immunostaining

In order to check the expression of CXCR4 in the tumor, semi-quantitative immunostaining was performed on the resected sections. The expression of CXCR4 was only moderate and homogenously distributed throughout the tumor (Fig-6). This was in agreement with tracer uptake seen in both ex-vivo biodistribution and PET studies.
Figure-5: 60-min ex-vivo biodistribution data of N-[\textsuperscript{11}C]methyl-AMD3465 in C6 glioma-rats. Wister rats were either untreated (control) or pretreated with 30 mg/kg AMD3100.8HCl 30 minutes before tracer injection. Significant differences between groups are indicated with * (p<0.05), ** (p<0.01), or *** (p<0.005). Data represent mean ± SD of the SUV (n=6).

PET imaging

Representative PET images of a control animal and an animal pretreated with AMD3100.8HCl are presented in Fig-7. Tracer uptake in the tumor is clearly visible in control animals, but not in pretreated animals. The time-activity curves (TACs) of the tracer accumulation in the tumor in control animals show a peak within 5 min after injection, followed by an exponential wash-out. Curve–fitting analysis showed a two-phase tumor clearance with half-lifes of 8±2 min (11%) and 115±1 min (89%). Tumor wash-out was significantly faster (p<0.05) in AMD3100-pre-treated animals, with half-lifes of 6±2 min (13%) and 60±3 min (87%). In one control animal, the tumor was necrotic and consequently a strong difference in tracer uptake between the tumor rim and the necrotic region was observed (SUV 0.65 and 0.23, respectively) this animal was excluded from all other calculations. ROIs were drawn manually on the tumor region, and TACs were generated for the total time of the scan (60 min). All values were converted to SUV. The tumor uptake of N-[\textsuperscript{11}C]methyl-AMD3465 (SUV 1.08±0.08, 5-60 min) as determined by PET was significantly reduced when animals were pretreated with non-radioactive AMD3100 (0.57±0.02, p<0.05).
Figure-6: Immunostaining showing CXCR4 receptor expression in a C6 tumor: a) CXCR4 positive staining, b) Negative staining without primary antibody. c) HE staining of the tumor section. The expression of the CXCR4 was found to be homogenous and the tumor shows normal morphology. All images were acquired at 10x magnification.

In addition, the area under the time/activity curve (AUC) of the tumor was also significantly reduced in tumors of animals that were pretreated with AMD3100 (Plerixafor) (51±4 vs 29±5, \( p<0.05 \)).

Figure-7: N-[\(^{11}\)C]methyl-AMD3465 PET images of rats bearing a C6 glioma tumor. The images represent a coronal view of the 0-60 min summed frames. a) Untreated control rat. b) Rat pretreated with 30 mg/kg AMD300.8HCl 30 min before tracer injection. c) A control animal with clear necrosis within the C6 tumor. The Arrows indicate the position of the tumor. d) Time activity curves of the tumor of control animals (n=6) and pretreated with 30 mg/kg AMD3100.8HCl (n=6). Significant differences are indicated by * (\( p<0.05 \)).
Supplement Figure 1: Synthesis of AMD4365 and N-methyl-AMD3465: Reagents and conditions (i) ethyl trifluoroacetate, triethylamine, MeOH, room temperature, overnight (ii) α,α-dibromoxylene, K$_2$CO$_3$, CH$_3$CN, reflux, over-night (iii) 2-picolamine, K$_2$CO$_3$, CH$_3$CN, reflux, 3 h. (iv) NaBH(OAc)$_3$, ClCH$_2$CH$_2$Cl, 30% formaldehyde, room temperature, overnight. (v & vi) K$_2$CO$_3$, MeOH, reflux, 3 h.

**DISCUSSION**

In this study, we evaluated N-[¹¹C]methyl-AMD3465 as a potential PET ligand for CXCR4 receptor imaging. N-[¹¹C]methyl-AMD3465 was prepared by N-methylation of the trifluoroacetyl protected precursor. First the labeling was attempted by methylation with [¹¹C]CH$_3$I under basic conditions. However, the presence of base (100 µL, 10mM NaOH) during the labeling of N-[¹¹C]methyl-AMD3465 resulted in deprotection of the cyclam ring and increased the formation of undesired byproducts; also the [¹¹C]CH$_3$I is less reactive than [¹¹C]CH$_3$OTf. Thus, the labeling of N-[¹¹C]methyl-AMD3465 was carried with [¹¹C]CH$_3$OTf in absence of base. The desired product was formed in acetone, but not in ethanol or acetonitrile. The radiolabeling reaction was further optimized for the amount of precursor, temperature, reaction time and amount of base. Radiochemical yields increased with temperature, but decreased again if the temperature was increased above 80°C, probably due to instability of the precursor or evaporation of [¹¹C]CH$_3$OTf or the solvent. N-[¹¹C]methyl-AMD3465 showed good in-vitro stability towards human
liver microsomes and rat plasma. The tracer is less hydrophilic (-0.86±0.09) than the related $^{64}$Cu and $^{99m}$Tc complexes of AMD3100 and AMD3465, which is likely due to the additional positive charge of the metal ions [10,17,19]. However, N-$^{[11]}$C]methyl-AMD3465 still shows fast renal clearance of unbound tracer and low levels of nonspecific binding in non-target tissues.

The methylation of the secondary amine in the linker between the cyclam and the pyridine ring resulted in a slight reduction in the binding affinity of AMD3465 towards CXCR4 receptors, but its IC$_{50}$ value is still lower (0.45±0.09 µM) than that of the drug AMD3100 (1.5±0.07 µM) [23]. The presence of transition metals resulted in a significant increase in the cellular uptake of the tracer. In particular, Cu$^{2+}$, Zn$^{2+}$ and Ni$^{2+}$ increased the uptake of the tracer. Incorporation of the transition metal ions in the cyclam ring of N-$^{[11]}$C]mehtyl-AMD3465 enhances the interaction of the tracer with the carboxylic group of aspartate 262 of CXCR4 receptor, which is located at the extracellular part of trans-membrane domain-VI in the receptor binding pocket. In contrast, Pd$^{2+}$ and Rh$^{4+}$ caused a significant decrease in tracer uptake, which might be due to the deviations in the planar structure of the cyclam when a complex is formed with these larger ions. At physiological pH, the cyclam has an overall charge of +2 and can accommodate its most stable conformation called trans-III type (R,R,S,S). In this conformation, the cyclam can readily form three hydrogen bonds with carboxylic group of aspartate-171 and aspartate-262 in binding domains IV and VI of CXCR4, respectively [23,24]. When the cyclam forms complexes with Cu$^{2+}$, Zn$^{2+}$ and Ni$^{2+}$, it can also adopt the trans-III conformation and as a result these complexes shows stronger binding to aspartate-262. In contrast, complexes with larger metal ions cannot adopt the optimal arrangement of the cyclam ring and as a result the affinity towards aspartate-171 and aspartate-262 is reduced, resulting in faster dissociation kinetics [24]. In our previous study, we found a similar reduction in binding affinity when the cyclam AMD3100 was labeled with the relatively large metal complex $[^{99m}\text{Tc}]$pertechnetate [10].

In-vitro binding studies in C6 and PC-3 cells showed specific binding, followed by rapid internalization of the tracer. Internalized tracer remained trapped inside the cells for the duration of the experiment. The cellular accumulation of the tracer can
therefore at least partly be ascribed to receptor mediated endocytosis and binding to cytosolic proteins [26]. The biphasic kinetics of the tracer in tumor TAC (both before and after receptor blocking) may be related to its being internalized, the extracellular pool showing a rapid kinetics and the intracellular pool showing a slow kinetics. Naturally C6 cells overexpress CXCR4 receptors both in normal and malignant condition [27].

Both PET and biodistribution studies showed specific binding in C6 tumors and also in organs with high expression of CXCR4, such as lymph node, bone marrow, spleen, thymus, adrenal glands, and liver. The tracer uptake in tumor and other organs was significantly reduced in blocking studies, in which the CXCR4 receptors were saturated by an excess of the selective antagonist AMD3100, indicating that the tracer shows specific binding to the receptor.

Previously, we investigated the bicyclam $[^{99m}\text{Tc}]\text{O}_2$-AMD3100 as a SPECT agent for CXCR4 imaging in nude mice with PC-3 tumor xenografts, with similar CXCR4 expression as the tumor model in the present study [10]. When compared to N-$[^{11}\text{C}]$methyl-AMD3465, the technetium-99m labeled tracer showed reduced binding affinity and faster washout kinetics. Between these tracers, N-$[^{11}\text{C}]$methyl-AMD3465 is clearly the superior imaging probe. Moreover, PET is a more sensitive technique and more suitable for absolute quantification of the receptor expression, which also favors N-$[^{11}\text{C}]$methyl-AMD3465 over $[^{99m}\text{Tc}]\text{O}_2$-AMD3100.

Besides N-$[^{11}\text{C}]$methyl-AMD3465, several other promising PET probes for CXCR4 imaging have been developed [28]. Especially, the $^{64}\text{Cu}$ labeled AMD3100 and AMD3465 derivatives showed very promising results with high tumor uptake (~10-96 % ID/g, in mice). The uptake values are very impressive, although it should be emphasized that these agents were tested in transgenic tumor models, in which CXCR4 was highly overexpressed [17,19,25]. Immunohistochemistry showed that CXCR4 is homogenously expressed in the C6 tumors used in our study, although the expression levels are only moderate. Since it is difficult to compare tracers that have been evaluated in transgenic CXCR4 overexpressing models with tracers that have been tested in tumor models with natural CXCR4 expression, it is unlikely to compare our results with previously reported data for $^{64}\text{Cu}$-labeled tracers and
consequently future studies with head-to-head comparison of these tracers are warranted.

An important difference between N-[^11]C)methyl-AMD3465 and ^64Cu-labeled AMD3465 and AMD3100 is the isotope applied for labeling of the CXCR4 antagonists. When CXCR4 receptors internalize, the ^64Cu-labeled tracers are transported into the cell. Since ^64Cu is a residualizing isotope, the internalized isotope remains trapped in the cell. In contrast, the internalized ^11C-labeled compound will be excreted again. Consequently, the ^64Cu accumulation will be enhanced due to internalization of the receptor, but not the ^11C accumulation. The uptake of ^64Cu-labeled tracers may therefore reflect receptor internalization rather than receptor expression. Another important issue is the longer half-life of ^64Cu (13 h) compared to ^11C (20 min). Consequently, distribution of the ^64Cu-labeled tracers to institutions close to the production site is possible. Unfortunately, currently the number of sites that produce ^64Cu is still limited. In contrast, N-[^11]C)methyl-AMD3465 cannot be distributed, as an on-site cyclotron is required for its production. Therefore, selection of the tracer of choice may be determined on local availability of the PET isotope, rather than on the imaging properties. Besides availability, the imaging properties of the isotopes may determine tracer selection. ^11C is a pure positron emitter, whereas ^64Cu decay comprises only 17% positron emission, which results in a poorer signal-to-noise ratio for ^64Cu. Moreover, the small percentage of positron decay, the almost 40% concomitant β^- decay (which can be used for treatment) and the longer half-life of ^64Cu will result in a substantially higher radiation burden to the patient. On the other hand, the mean positron energy of ^11C is higher than that of ^64Cu, which can result in a reduction of the spatial resolution that can be obtain in high-resolution small animal PET imaging and autoradiography. So, for institution with a cyclotron on-site N-[^11]C)methyl-AMD3465 could be an attractive candidate PET tracer for imaging of CXCR4.
CONCLUSION

N-[\(^{11}\)C]methyl-AMD3465 shows promising properties as a PET tracer for the detection and quantification of CXCR4 receptors in tumors. The tracer can be readily prepared and shows good stability. Both *in-vitro* and *in-vivo* data indicated selective and specific binding towards CXCR4 receptors. PET data demonstrated the feasibility of *in-vivo* imaging of CXCR4 receptors in C6 tumors, which naturally express the receptor. Taken together, these results warrant further evaluation of N-[\(^{11}\)C]methyl-AMD3465 as a PET tracer for CXCR4 receptor imaging.

ACKNOWLEDGMENTS

We would like to thanks Jürgen Sijbesma, Willem Jan Kuik, and Alexandre Shoji for providing the excellent help during the experiment. This work was supported in part by grants from Jan Kornelis De Cock-Stitching. No other potential conflict of interest relevant to this article was reported.
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