Development and evaluation of molecular imaging probes for CXCR4 mediated chemotaxis and tumor infiltration of activated T-Cells
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Chapter 4:

N-[\textsuperscript{\textit{11}}C]methyl-AMD3465 PET as a tool for \textit{in-vivo} Measurement of Chemokine Receptor 4 (CXCR4) Occupancy by the Therapeutic Drugs

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ABSTRACT

CXCR4 is an α-chemokine receptor overexpressed in many cancers and a potential drug target. We have recently developed the tracer N-[11C]methyl-AMD3465 for imaging of CXCR4 expression by positron emission tomography (PET). Here, we investigated the pharmacokinetics of N-[11C]methyl-AMD3465 in rats bearing a C6 tumor and assessed whether the CXCR4 occupancy by the drug Plerixafor® can be measured with this PET tracer. **Methods:** A subcutaneous C6 tumor was grown in male Wistar rats. A dynamic N-[11C]methyl-AMD3465 PET scan with arterial blood sampling was performed in control rats and rats pretreated with Plerixafor® (30 mg/kg, s.c). The distribution volume (V_T) of the tracer was estimated by Logan graphical analysis and compartment modeling with a 2 tissue reversible compartment model (2TRCM). The non-displaceable binding potential (BP_{nd}) was estimated with the 2TRCM. Next, CXCR4 receptor occupancy of different doses of the drug Plerixafor® (0.5 - 60 mg/kg AMD3100.8HCl) was investigated. **Results:** The C6 tumor could be clearly visualized by PET in control animals. Pre-treatment with 30 mg/kg Plerixafor® significantly reduced tumor uptake (SUV 0.65±0.08 vs. 0.20±0.01, p<0.05). N-[11C]methyl-AMD3465 was slowly metabolized in-vivo, with 70±7% of the tracer in plasma still being intact after 60 min. The tracer showed reversible in-vivo binding to its receptor. Logan graphical analysis could best be used to estimate V_T. Pre-treatment with 30 mg/kg Plerixafor® resulted in a significant reduction in V_T (0.69±0.10 vs. 0.34±0.13, p<0.05) and BP_{nd} (1.73±0.18 vs. 1.08±0.35, p<0.01). Receptor occupancy by Plerixafor® was dose-dependent with an in-vivo ED_{50} of 3.6±1.0 mg/kg. **Conclusion:** N-[11C]methyl-AMD3465 PET can be used to visualize CXCR4 receptor expression and to calculate receptor occupancy. V_T determined by Logan graphical analysis is a suitable parameter to assess CXCR4 receptor occupancy. This approach can easily be translated to humans and used for early drug development and optimization of drug dosing schedules.
INTRODUCTION

Chemokine receptor 4 (CXCR4) is a member of the α-chemokine receptor family, which is involved in maintaining body homeostasis, hematopoiesis, immune cell trafficking, and homing and retention of stem cells [1,2]. However, CXCR4 and its natural ligand Stromal Derived Factor-1α (CXCL12) also play a crucial role in the development, proliferation and metastasis of cancer [3]. Elevated expression of CXCR4 has been observed in more than 20 different human tumor types [4-6] and the level of CXCR4 expression was associated with tumor grade and poor prognosis [7-8]. CXCR4 has been considered as a target for (adjuvant) cancer therapy [9,10]. Inhibition of CXCR4-mediated signaling has therapeutic efficacy by itself, but can also have a synergistic effect with other anti-cancer drugs, such as bortezomib, melphalan, doxorubicin, and dexamethasone [11].

In drug development, it is essential to know the kinetics and the in-vivo binding properties of a drug to its target at an early stage, preferably in animal studies or in early clinical trials. Usually, there is a threshold for the percentage of the receptors that have to be occupied by the drug in order to achieve a pharmacological effect. Currently, drug dosing regimens for patients are mainly based on the outcome of preclinical and phase I-II studies. However, these studies cannot elucidate the relationship between amount of drug administered and the occupancy of the receptors in-vivo.

Positron emission tomography (PET) can be used to noninvasively measure receptor occupancy in-vivo by comparing the uptake of a specific tracer in the tissue of interest before and after administration of one or more doses of the drug under investigation. These studies can answer many critical questions, such as whether the drug has reached its target, the level of receptor occupancy after administration of a therapeutic dose and the duration of receptor binding by the drug. With this information, effective dosing regimens can be designed for the expensive phase 3 clinical trials.

Recently, we have developed N-[\(^{11}\)C]methyl-AMD3465 as a radiotracer for PET imaging of CXCR4 receptors [12]. The aim of this study is to investigate various methods for quantification of CXCR4 availability using N-[\(^{11}\)C]methyl-AMD3465 PET
in immune-competent tumor-bearing rats. We also investigated whether this approach can be applied to measure CXCR4 receptor occupancy of the drug Plerixafor® (AMD3100.8HCl). Till date, no report has been published on the estimation of CXCR4 receptor occupancy by PET.

**MATERIALS AND METHODS**

**General**

All reagents and solvents were obtained from commercial suppliers and used without further purification. AMD3100 octahydrochloride (Plerixafor®) was prepared as previously described [13,14]. A stock solution of Plerixafor® was prepared in phosphate buffered saline (PBS) and neutralized with 1M NaOH. N-[\(^{11}\)C]methyl-AMD3465 was prepared as previously described [12]. C6 rat glioma cells (ATCC, Manassas, VA) were cultured in monolayers in Dulbecco's Modified Eagle Medium, supplemented with 10 % fetal calf serum. Cells were maintained in a humidified atmosphere with 5 % CO\(_2\) at 37\(^\circ\)C.

**Animal model**

All animal experiments were performed in compliance with the Dutch law on Animal experiments. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Groningen. Male Wistar rats (n=34, 300±20g, Harlan) were maintained at a 12 h day and 12 h night regime and fed standard laboratory chow ad libitum. Rat C6 glioma cells (3 million) in a mixture of Matrigel and complete medium (1:1 v/v) were subcutaneously injected into the right shoulder of the animal. Solid tumors (350±20 mg) were allowed to grow for 5-6 days before the animals underwent PET experiments. The study was divided into two parts: first different pharmacokinetic modeling approaches to quantify N-[\(^{11}\)C]methyl-AMD3465 uptake were evaluated, then the CXCR4 receptor occupancy of the drug Plerixafor was investigated.

**Small animal PET**

Animals (n=6) were anesthetized with a mixture of isoflurane/air (5% induction and 2% maintenance, Teva Pharmachemie, The Netherlands). A cannula was placed in the femoral artery for rapid blood sampling and another cannula was inserted in
the femoral vein for tracer injection. Two animals were simultaneously placed in the PET camera (Focus-220, Siemens-Concorde). A transmission scan with a Co-57 point source was made for attenuation and scatter correction. After the transmission scan, 30±5 MBq of N-[11C]methyl-AMD3465 in a volume of 1 mL was injected as a slow bolus over a period of 1 min using a Harvard-style pump. The PET scan was started when the tracer started to enter the body of the first animal. The second animal was injected 16 min later. A 76-min list mode protocol was used for data acquisition. A series of 15 blood samples (0.10-0.15 mL) was drawn at 10, 20, 30, 40, 50, 60, 90, 120, 180, 300, 450, 600, 900, 1800 and 3600 s after tracer injection. After a blood sample was taken, 0.15 mL of heparinized saline was injected via the artery cannula to prevent hypovolemia and large changes in blood pressure. From each blood sample, 25 µL was taken for radioactivity measurements, using a gamma-counter. The remainder of the sample was centrifuged (Eppendorf centrifuge, 5 min at 13,000 rpm) to separate plasma from blood cells and radioactivity in 25 µL of plasma was measured. The radioactivity measurements in blood and plasma were used to construct the plasma and whole blood input functions for pharmacokinetic modeling.

To examine specific binding, 6 rats were pretreated with Plerixafor® (30 mg/kg, s.c.), 30 min before the tracer injection. Scanning was performed as described above. After the scan, the animals were terminated under deep anesthesia.

List mode data of the emission scan was separated into 21 frames (6x10 s, 4x30 s, 2x60 s, 1x120 s, 1x180 s, 4x300 s, and 3x600 s). Emission sinograms were iteratively reconstructed (OSEM2D, 16 subset, 4 iterations) after being normalized and corrected for attenuation, scatter and radioactive decay. The data sets consisted of 95 slices, with a slice thickness of 0.8 mm and an in-plane 128 x 128 image matrix with a pixel size of 1.1 mm. Images were smoothed with a 1-mm Gaussian filter. Volumes of interest (VOIs) of the tumor were drawn on the summed PET images using a threshold of 75% of the maximum uptake in the tumor. For each VOI, a time activity curve (TACs) was calculated using standard software (Inveon Research Workplace, Siemens-Concorde). Tracer uptake was normalized to the injected tracer dose and body weight and is presented as Standardized Uptake Value (SUV).
**In-vivo Metabolite analysis**

For metabolite analysis (n=6), a series of 0.5 – 0.8 mL arterial blood samples was drawn at 5, 15, 30, 40 and 60 min after tracer injection. Blood samples were centrifuged at 13,000 rpm for 2 min. Plasma was collected, 0.1 mL of 70% H$_3$PO$_4$ was added and the solution was vortexed for 30 s. The mixture was centrifuged for 2 min at 13,000 rpm and the clear supernatant was collected. The supernatant was analyzed by HPLC, using a µBondapak column [7.8 x 300 mm, Waters] and water/acetonitrile (90/10 v/v) as the eluens. The flow-rate was set at 3 mL/min and samples were collected at time intervals of 30 s. The radioactivity in the collected samples was measured with a gamma-counter and the percentage of intact tracer was calculated. An average metabolite curve was generated by fitting the data points of all animals with an exponential function. Metabolite-corrected plasma curves were generated by correcting the plasma curves of the individual animals for the percentage of intact tracer, using the population-based metabolite curve. The metabolite-corrected plasma curves of individual animals were used as an input function curve for pharmacokinetic modeling.

**Graphical analysis**

Graphically analysis was performed on the TACs of the tumor, using the Logan and Gjedde–Patlak graphical methods. The whole blood and the metabolite-corrected plasma curves were used as input functions. Logan graphical analysis could describe the tracer kinetics best and was therefore used to calculate the total volume of distribution (V$_T$) of the tracer in the tumor.

**Compartment modeling**

Tracer kinetics of N-[¹¹C]methyl-AMD3465 was analyzed by compartmental modeling using the 1-tissue compartment model (1TCM) and the 2-tissue reversible compartment model (2TRCM). The whole blood curve and the metabolite-corrected plasma curve were used as input functions; the blood volume fraction (V$_b$) was not fixed. Inveon workstation software (Siemens) was used to estimate the model parameters (rate constants, V$_b$). Akaike information criterion values indicated that the 2TRCM fits the tracer kinetics in the tumor best. Tracer uptake
was expressed as $V_T$ and non-displaceable binding potential ($BP_{nd}$), which were defined as [15-16].

$$V_T = \frac{K_v}{k_2} (1 + BP_{nd}) \quad BP_{nd} = \frac{k_3}{k_4}$$

**Receptor occupancy**

The CXCR4 receptor occupancy was investigated in C6 tumor-bearing rats that were subcutaneously injected with different doses of Plerixafor® (0.5, 3, 10, 30 and 60 mg/kg, n=3/dose). After 30 min, N-[11C]methyl-AMD3465 was administered and a 60 min dynamic PET scan with arterial blood sampling was performed as described above. Logan graphical analysis and compartment modeling with the 2TRCM were used to determine the $V_T$ of the tracer in the tumor. The occupancy of the drug was calculated from the average $V_T$ of control animals ($V_T$ (baseline)) and the $V_T$ of animals that were administered the drug ($V_T$ (drug)). The occupancy of Plerixafor® was estimated according to the formula:

$$Occupancy = \left( \frac{V_T(\text{baseline}) - V_T(\text{drug})}{V_T(\text{baseline})} \right) \times 100$$

Likewise, the occupancy was estimated using the $BP_{nd}$, instead of $V_T$. In the formula for the occupancy, non-specific binding was not taken into account. The $ED_{50}$ of Plerixafor® and the percentage of nonspecific binding of the tracer were estimated using a non-linear regression method by plotting the $V_T$ or the BP versus the dose of Plerixafor® using the equation:

$$Occupancy = \min + \left( \frac{\max - \min}{1 + \left[ \frac{[\text{AMD3100.8HCl}]}{ED_{50}} \right]^{Hillslope}} \right)$$

In this equation, “max” and “min” represent the occupancy at an infinite concentration of the drug (maximum saturation) and the occupancy in the absence of the drug, respectively. Thus, the percentage of nonspecific binding can be calculated from this formula as 100% minus “max”. In order to calculate the occupancy of the drug Plerixafor® more accurately, the measured $V_T$ values were
corrected for nonspecific binding and used to estimate the percentage of occupancy using the formula:

\[
Occupancy = \left( \frac{V_t(base) - V_t(nonspecific) - [(V_t(drug) - V_t(nonspecific))]}{V_t(base) - V_t(nonspecific)} \right) \times 100
\]

In the same way, the receptor occupancy was calculated by using \(BP_{nd}\), instead of \(V_t\).

**Statistical Analysis**
All results are expressed as mean ± standard deviation. Statistical analyses were performed using the two-sided unpaired students’ t-test in GraphPad Prism 5. A probability (\(p\)) value <0.05 was considered statistically significant.

**RESULTS**

**PET imaging**
The N-[\(\text{\textsuperscript{11}}\text{C}\)]methyl-AMD3465 PET images (0-60 min) of a control rat with a C6 tumor and a rat pretreated with 30 mg/kg Plerixafor® are represented in Fig-1. The C6 tumor is clearly visible in the control animal, but not in the Plerixafor® treated animal, indicating that the drug is able to compete with the PET tracer for the binding site of CXCR4. Pre-treatment with 30 mg/kg Plerixafor® significantly reduced the tracer uptake the in tumor (SUV 0.65±0.08 vs. 0.20±0.01, \(p<0.05\)).

**Tracer kinetics**
Fig-2 presents the average TACs of N-[\(\text{\textsuperscript{11}}\text{C}\)]methyl-AMD3465 in the tumor of control and Plerixafor® pre-treated animals. Tracer uptake in the tumor reached a maximum at 30 s after injection, followed by a bi-exponential clearance in both groups. The clearance half-lifes of the tracer from the tumor were 10±1 min (10%) and 129±9 min (90%) in control rats and 8±3 min (14%) and 68±5 min (86%) in Plerixafor®-treated animals. Pretreatment with Plerixafor® resulted in significantly (\(p<0.05\)) lower activity levels in the tumor of pre-treated animals than in tumors of control animals beyond 5 min after tracer injection (Fig-2a). The clearance of the tracer from plasma was not affected by the drug treatment (Fig-2b), as plasma activity was not significantly different between control animals and Plerixafor® pretreated rats at any time point.
Figure-1: Coronal PET images (0-60 min) of C6 tumor-bearing Wistar rats. a) Control animal with the tumor and heart in the field of view. b) Animal pre-treated with AMD3100.8HCl (Plerixafor®: 30 mg/kg), 30 min before the tracer injection. The arrow indicates the position of the tumor; Sg: Salivary gland, Ln: Lymph node and Ht: Heart.

Metabolite analysis

HPLC analysis of plasma samples showed the formation of one radioactive metabolite that was more hydrophilic than N-[11C]methyl-AMD3465 (retention times: N-[11C]methyl-AMD3465 10 min, radioactive metabolite 5 min; Fig-3a). The tracer was slowly metabolized in vivo and metabolism was highly reproducible between animals. The percentage of intact tracer decreased from 98±2 % at 5 min to 70±7 % at 60 min (Fig-3b).

Graphical analysis

Fig-4 shows representative examples of a Logan and a Patlak plot of N-[11C]methyl-AMD3465 uptake in the tumor. The tracer kinetics showed a better fit with Logan graphical analysis (R²=0.99±0.08) than with Patlak analysis (R²=0.17±0.42), suggesting that the binding of N-[11C]methyl-AMD3465 to CXCR4 receptors is reversible. The Vr calculated by Logan graphical analysis was significantly lower in rats pretreated with Plerixafor® than in control rats (0.26±0.11 vs 0.78±0.16, p<0.05), indicating that the tracer displays specific binding to the CXCR4 receptor (Table-1).
Figure-2: A) Time-activity curves of the C6 tumor in Wistar rats (n=6). Animals were either untreated (Control) or injected with 30 mg/kg Plerixafor® 30 min before the tracer injection (Blocker). The uptake of the tracer is expressed as SUV. B) Kinetics of N-[11C]methyl-AMD3465 in plasma. Plasma data were corrected for the metabolites. Pre-treatment with Plerixafor® did not significantly affect the kinetics of the tracer in plasma. Error bars represent standard deviations. Statistically significant differences are indicated by * (p<0.05).

Table 1: Results of kinetic modeling of N-[11C]methyl-AMD3465 tumor kinetics in C6 glioma-bearing Wistar rats. Animals were either untreated (control) or treated with 30 mg/kg Plerixafor® 30 min prior to tracer injection. Data are presented as a mean ± standard deviation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=6)</th>
<th>Plerixafor® (n=6, 30 mg/kg)</th>
<th>Statistical Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Logan graphical analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VT</td>
<td>0.78±0.16</td>
<td>0.26±0.11</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>2TRCM compartment modeling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VT</td>
<td>0.77±0.10</td>
<td>0.34±0.13</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>BP</td>
<td>1.73±0.18</td>
<td>1.08±0.35</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Vb</td>
<td>0.06±0.07</td>
<td>0.05±0.01</td>
<td>NS</td>
</tr>
</tbody>
</table>

Compartment Modeling

TACs generated from the VOI of the tumor were analyzed with different reversible compartment models. The AIC values were 423 ± 28 and 296 ± 13 for the 1TCM and 2TRCM, respectively, indicating that tracer kinetics could be better fitted with the 2TRCM. Therefore, the 2TRCM was used to estimate the parameters K1, k2, k3, k4 and Vb, which were subsequently converted into VT and BPnd (Table-1). Animals treated with Plerixafor® showed a significantly lower BPnd than control animals (1.73±0.18 vs 1.08±0.35, p<0.01). Similarly, the VT was significantly reduced after pre-treatment with Plerixafor® (55%, p<0.05). Pre-treatment with Plerixafor did not
affected the curve fit, as tracer kinetics could still be fitted by the 2TRCM. Compartment modeling with the 2TRCM indicated that the estimated total blood volume ($V_b$) was not affected by Plerixafor® treatment (0.06±0.07 vs. 0.05±0.01 in control and drug-treated animals, respectively).

![Figure 3](image1.png)

**Figure-3:** *In-vivo* metabolite analysis (n=6). A) Chromatogram representing the plasma metabolite analysis by HPLC. B) Graph showing the percentage of intact tracer in plasma as a function of time. Error bars represent standard deviations.

![Figure 4](image2.png)

**Figure-4:** Representative graphical analyses of tracer uptake in the C6 tumor. A) Logan graphical analysis using a 10 min delay time. B) Patlak graphical analysis with a delay time of 20 min.

**Receptor occupancy**

The occupancy of CXCR4 receptors in the tumors (uncorrected for nonspecific binding), as estimated from the $V_T$ determined by compartment modeling with the 2TRCM, ranged from 6 % to 86 % for Plerixafor® doses between 0.5 to 60 mg/kg, respectively. The occupancies of the drug calculated from $V_T$ derived from Logan graphical analysis correlated well with those obtained with the 2TRCM ($R^2=0.99$).
The occupancy values of the drug, as calculated from the BP_{nd} obtained from the 2TRCM, were lower than those calculated from the V_t. The occupancy calculated from BP_{nd} showed only a moderate correlation with the occupancy determined from V_t (r^2=0.77).

Non-linear regression analysis was used to estimate the ED_{50} of Plerixafor® in the living animal. Thus, the \textit{in-vivo} ED_{50} of the drug were 3.7±0.1, 3.7±0.0 and 11.8±7.6 mg/kg when the occupancy was estimated from the V_t derived from Logan graphical analysis, the V_t derived from the 2TRCM and the BP_{nd}, respectively (uncorrected for nonspecific binding). The non-linear regression of occupancy data derived from the BP_{nd} did not reach a statistically significant curve fit (p=0.17) and therefore the ED_{50} estimated by this method should be considered as unreliable.

**Figure-5:** Receptor occupancy graphs: A) The receptor occupancy was estimated by non-linear regression analysis. The tumor distribution volume (V_t) and the non-displaceable binding potential (BP_{nd}), uncorrected for nonspecific binding, were used to calculate the percentage of CXCR4 receptor occupancy for the drug Plerixafor®. B) Estimation of the ED_{50} from the receptor occupancy after correction for nonspecific binding.

Extrapolation of the occupancy curves to an infinite drug dose revealed that nonspecific binding of the tracer in the tumor was approximately 14%, irrespective of the method that was used to determine the occupancy (Fig-5a, Table-2). This estimation of unsaturable tracer uptake allowed a more accurate estimation of the receptor occupancy and ED_{50} by taking nonspecific binding of the tracer into account. After correction for nonspecific binding, the estimated receptor occupancy reached almost 100 % when calculated from the V_t from Logan analysis or compartment modeling using the 2TRCM.
Table 2: Percentage of receptor occupancy calculated from the volume of distribution (VT) and non-displaceable binding potential (BP_{nd}) obtained from 2TRCM and Logan graphical analysis after correction for non-specific binding. The C6 tumors bearing Wistar rats were treated with different concentrations of Plerixafor 30 min before tracer injection. All data are expressed as the mean ± standard deviation.

<table>
<thead>
<tr>
<th>Drug Concentration (n=3, mg/kg)</th>
<th>Occupancy (%) (VT – Logan)</th>
<th>Occupancy (%) (VT – 2TRCM)</th>
<th>Occupancy (%) (BP_{nd} - 2TRCM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>15±1</td>
<td>12±4</td>
<td>1±3</td>
</tr>
<tr>
<td>3</td>
<td>56±7</td>
<td>20±6</td>
<td>7±5</td>
</tr>
<tr>
<td>10</td>
<td>98±2</td>
<td>99±2</td>
<td>48±3</td>
</tr>
<tr>
<td>30</td>
<td>99±1</td>
<td>100±1</td>
<td>67±7</td>
</tr>
<tr>
<td>60</td>
<td>100±2</td>
<td>100±2</td>
<td>89±2</td>
</tr>
<tr>
<td>ED_{50} (mg/kg)</td>
<td>3.6±1.0</td>
<td>4.2±0.9</td>
<td>12.6±6.4*</td>
</tr>
<tr>
<td>Non-specific binding</td>
<td>14±1 %</td>
<td>14±0 %</td>
<td>13±29 %*</td>
</tr>
</tbody>
</table>

*The non-linear regression curve fit used to estimate these parameters did not reach statistical significance.

In contrast, the occupancy calculated from the BP_{nd} reached only 90% at the maximum dose of 60 mg/kg. The estimated ED_{50} values were 3.6±1.0, 4.2±0.9 and 12.6±6 mg/kg when estimated from nonspecific binding corrected VT (Logan), VT (2TRCM) and BP_{nd} respectively (Fig-5b).

DISCUSSION

In this study, we showed that occupancy of CXCR4 receptors by the drug Plerixafor® can be measured in-vivo in C6 glioma-bearing rats, using PET and kinetic modeling. Plerixafor® is a potent and selective CXCR4 receptor antagonist, which was originally developed as an anti-HIV drug, but nowadays it is also used as a drug in adjuvant cancer therapy and to stimulate the mobilization of hematopoietic stem cells from bone marrow for autologous transplantation. Using N-[\textsuperscript{11}C]methyl-AMD3465 PET, we assessed CXCR4 receptor occupancy by the drug and calculated its ED_{50} in-vivo.

Pre-treatment with Plerixafor® (30 mg/kg) resulted in a significant reduction in the uptake of the tracer in the tumor, indicating that N-[\textsuperscript{11}C]methyl-AMD3465 shows specific binding to CXCR4. Tracer kinetics in blood and plasma were not affected by pre-treatment with Plerixafor®. In-vivo metabolite analysis of plasma showed that the tracer was slowly metabolized in-vivo, as approximately 70% of N-[\textsuperscript{11}C]methyl-
AMD3465 was still intact 1 h after tracer injection. Only one polar radioactive metabolite was detected in plasma (Fig-3a). MetaPrint2D software (Cambridge) predicted that this metabolite of N-[\textsuperscript{11}C]methyl-AMD3465 was either formed by dealkylation at an amine group in the cyclam ring or hydroxylation of a secondary nitrogen atom in the cyclam. Since the cyclam ring is involved in ligand binding to the active site of the receptor, it seems unlikely that the radioactive metabolite has significant affinity for CXCR4 (data not shown).

Logan and Patlak graphical analysis of the TACs obtained from the tumor could be better fitted by Logan than Patlak analysis. This indicates that the binding of N-[\textsuperscript{11}C]methyl-AMD3465 to the CXCR4 receptors is reversible, which was further supported by compartmental analysis. Different compartmental models were used to fit the PET data and the best model fit was obtained for 2TRCM. Pretreatment with the drug Plerixafor\textsuperscript{®} resulted in a significant decrease in BP\textsubscript{nd} and V\textsubscript{T}, indicating that specific tracer uptake could be inhibited by saturation of the CXCR4 receptor. Furthermore, estimation of total blood volume (V\textsubscript{b}) was included in the analysis, since it can be affected by treatment with drugs [17]. In this study, however, we did not find any effect of Plerixafor\textsuperscript{®} on tumor blood volume.

PET is a noninvasive imaging technique, which can provide insight into the relationship between receptor occupancy and drug efficacy, provided a suitable radioligand is available [18]. In the second part of our study, the feasibility of determining CXCR4 receptor occupancy by Plerixafor\textsuperscript{®} using N-[\textsuperscript{11}C]methyl-AMD3465 PET was assessed. For this purpose, both V\textsubscript{T} and BP\textsubscript{nd} values obtained from Logan and 2TRCM analysis were used to estimate the CXCR4 receptor occupancy. For occupancy measurements, a reliable fit with high reproducibility is necessary. Our data showed that V\textsubscript{T} obtained from either Logan analysis or from 2TRCM analysis were highly correlated and gave a similar estimation of receptor occupancy. In contrast, V\textsubscript{T} showed only a moderate correlation with BP\textsubscript{nd} and occupancy values obtained from the BP\textsubscript{nd} could not be reliably fitted to calculate the ED\textsubscript{50}. Theoretically, BP could give more accurate results, because it only relies on the receptor binding parameters k\textsubscript{3} and k\textsubscript{4}, whereas V\textsubscript{T} can also be affected by tracer delivery and tissue clearance. In theory one would therefore expect that BP\textsubscript{nd}
is the parameter of choice to calculate the receptor occupancy, since it is independent of physiological parameters that could be affected by drug pretreatment, like blood flow, perfusion etc. However, $BP_{nd}$ relies on the accurate estimation of the $k_3$ and $k_4$ values with the 2TRCM. Since the 2TRCM requires simultaneous estimation of 5 parameters ($K_1$, $k_2$, $k_3$, $k_4$ and $V_b$), curve fitting may sometimes be difficult and the estimated values for $k_3$ and/or $k_4$ can be highly sensitive to noise, especially when the number of available receptors is low, for example in case of high receptor occupancy. Logan graphical analysis was a more stable modelling approach, which can be used even when the 2TRCM does not fit properly.

Due to lack of a specific reference region for estimation of nonspecific binding, we calculated the nonspecific binding in the tumor by extrapolating the occupancy curves to an infinitely high drug dose. About 14% of tracer uptake in control tumors was due to non-specific binding, irrespective of whether $V_T$ or $BP_{nd}$ were used to calculate the occupancy. This low percentage of non-specific binding leaves a sufficiently large dynamic range to distinguish differences in receptor occupancy levels. We used $V_T$ calculated from Logan analysis and from 2TRCM analysis to estimate the $ED_{50}$ of the drug Plerixafor® $in-vivo$. Both approaches gave comparable results. In contrast, when $BP_{nd}$ values were used to estimate the $ED_{50}$, non-linear regression of the data did not give a statistically significant fit and consequently the calculated $ED_{50}$ values were unreliable. This poor curve fit was probably caused by $BP_{nd}$ being more sensitive to noise than $V_T$, resulting in poor estimates of the occupancy by this method. Therefore, our results suggested that estimation of $ED_{50}$ is more reliable when CXCR4 receptor occupancy is calculated from the $V_T$ obtained by either Logan or 2TRCM analysis than when occupancy is calculated from $BP_{nd}$. When receptor occupancy was corrected for nonspecific binding, almost 100% occupancy was obtained at a doses $\geq 10$ mg/kg, if occupancy was calculated from $V_T$ (Logan and 2TRCM). Correction of the $BP_{nd}$ for nonspecific binding did not improve the estimation of the $ED_{50}$, as non-linear regression failed to give a statistically significant curve fit.
A limitation of this study is that the effect of the endogenous ligand CXCL12 was not assessed. The effect of changes in binding of CXCL12 to CXCR4 on occupancy calculations is probably negligible, because all animals likely had comparable CXCL12 levels as they received the same treatment. Another limitation is that the C6 tumors express both CXCR4 and CXCR7 [19]. Both CXCL12 and Plerixafor® can bind to both CXCR4 and to CXCR7 [20]. Therefore, both CXCR4 and CXCR7 may have contributed to the occupancy measured in this study. These issues need to be addressed in future studies.

**CONCLUSION**

We have demonstrated that N-[\(^{11}\)C]methyl-AMD3465 can be used to quantify CXCR4 receptor occupancy in tumors with PET. Tracer kinetics can be easily quantified by Logan graphical analysis, which gives comparable estimates of V_1 as compartment modeling using the 2TCRM. Estimation of the BP_{nd} from the 2TCRM appears to be sensitive to noise. N-[\(^{11}\)C]methyl-AMD3465 PET seems to be a useful tool to establish the relationship between drug dose and CXCR4 receptor occupancy *in vivo*. This technique could easily be translated to applications in humans, like patient-tailored, individualized therapy monitoring and development of new drugs for CXCR4 receptors.
REFERENCES


