Targeting EGFR and VEGFR2 Tyrosine Kinases with Positron Emission Tomography – Evaluation of two Radiotracers

Erik Samén

Stockholm 2014
Cover illustration:
Thesis in flower shape. Each petal and sepal representing important elements of the thesis.

All previously published papers were reproduced with permission from the publisher.

Published by Karolinska Institutet.

Printed by University Service US-AB, Stockholm, Sweden

© Erik Samén, 2013
ABSTRACT

Receptor tyrosine kinases (RTKs) are commonly involved in the development, growth and spread of cancer. Targeted therapy with tyrosine kinase inhibitors (TKIs) has proven a successful treatment strategy against cancers in which growth is dependent on the expression of these receptors. Increased effectiveness of treatment can potentially be achieved by individual characterizations of the disease, enabling tailoring of the therapy directed at the specific targets found.

Positron emission tomography (PET) is a non-invasive imaging technique that allows characterization of biochemical processes and quantification of targets such as RTKs. In PET, the distribution of radiolabeled molecules in the body is traced by the emission of photons produced after the decay of the radionuclide that is incorporated in the tracer molecule. Drugs and other xenobiotics are often metabolized in the body to facilitate excretion. A PET tracer can be metabolized into radioactive metabolites, which often display pharmacokinetic behaviors different than that of the parent molecule. It is therefore pivotal to characterize the metabolism of novel PET tracers before accurate estimations of biological target levels, based on radioactivity uptakes, can be performed.

This doctoral thesis focuses on the preclinical evaluation of two tracer molecules, $[^{11}\text{C}]$PD153035 and $[^{11}\text{C}]$PAQ, each targeting a specific RTK known to play important roles in cancerogenesis. Both tracers are based on TKIs and have been proven to be potent RTK inhibitors in vitro. In papers I and III, the in vitro and in vivo metabolism, respectively, of $[^{11}\text{C}]$PD153035 was investigated. We found that the tracer was extensively metabolized by cytochrome P450 enzymes into several different radioactive metabolites. Furthermore, the results indicate that the metabolism impairs quantification of the target RTK, the epidermal growth factor receptor (EGFR).

In papers II and IV the pharmacokinetics and angiogenesis detection properties of (R,S)-$[^{11}\text{C}]$PAQ and (R)-$[^{11}\text{C}]$PAQ were assessed in various models of cancer in mice. The results show that the tracer is metabolically stable and that areas with increased angiogenic activity, based on vascular endothelial growth factor receptor 2 expression (VEGFR2), can be visualized with PET. Uptake of radioactivity correlated well to areas with high expression of the receptor both with the labeled racemate and the R-isomer. In addition, high focal uptake was observed with (R)-$[^{11}\text{C}]$PAQ in lungs with metastases that exhibited high expression levels of the VEGFR2.

In summary, we conclude that $[^{11}\text{C}]$PD153035 is metabolized very rapidly in rat and that a similar metabolism in humans would imply serious limitations if the tracer is used in patient stratification for EGFR targeted therapy. (R)-$[^{11}\text{C}]$PAQ, on the other hand, is a promising tracer that, pending positive results in further validation studies, can prove to be a valuable tool for personalizing cancer treatment based on expression levels of VEGFR2.
To see a world in a grain of sand
and a heaven in a wild flower,
hold infinity in the palm of your hand
and eternity in an hour

From "Auguries of Innocence"
by William Blake (1757-1827)
LIST OF PUBLICATIONS


Publications that are not part of this thesis:


CONTENTS

INTRODUCTION

1 Imaging Techniques ................................................................. 1
   1.1 Positron Emission Tomography ............................................. 1
       1.1.1 Data Processing and Image Analysis ................................. 3
   1.2 Computed Tomography .......................................................... 4
   1.3 Magnetic Resonance Imaging ................................................... 5
   1.4 Combining Modalities ............................................................ 6
   1.5 Ex vivo Immunohistochemistry ................................................. 7
   1.6 Ex vivo Phosphor Imaging ....................................................... 8

2 PET Radiotracers ........................................................................... 8
   2.1 Tyrosine Kinase Inhibitors ....................................................... 8
       2.1.1 [\(^{11}\)C]PD153035 and Tracer Analogs ............................ 10
       2.1.2 [\(^{11}\)C]PAQ and Tracer Analogs ..................................... 11
   2.2 Antibodies, Proteins and Peptides ............................................. 12
   2.3 Radiolabeling with Carbon-11 ................................................. 13
       2.3.1 Specific Radioactivity .................................................. 14

3 Pharmacokinetics .......................................................................... 15
   3.1 Metabolism In vitro and In vivo ................................................ 15
       3.1.2 Metabolism of Tyrosine Kinase Inhibitors ......................... 16
   3.2 Biodistribution ........................................................................ 17
       3.2.1 Binding Properties ........................................................ 17
       3.2.2 Transport Proteins and Tyrosine Kinase Inhibitors .......... 19

4 Cancer .......................................................................................... 20
   4.1 Metastasis .............................................................................. 21
   4.2 Angiogenesis .......................................................................... 22
   4.3 Receptor Tyrosine Kinases ..................................................... 22
       4.3.1 Epidermal Growth Factor Receptor ............................... 23
       4.3.2 Vascular Endothelial Growth Factor Receptor 2 ........... 24
   4.4 Diagnosis with Positron Emission Tomography ....................... 25
   4.5 Tyrosine Kinase Inhibitors in Treatment .................................. 26
   4.6 Targeted Treatment and Personalized Therapy ......................... 28

5 Cancer models ............................................................................... 29
   5.1 Implants and Injection of Tumor Cells ..................................... 29
   5.2 Genetically Modified Animals ............................................... 29
   5.3 Animals and Ethical Considerations ......................................... 30

AIMS OF THE THESIS .................................................................... 31
RESULTS AND COMMENTS

In vitro Metabolism of [11C]PD153035 (Paper I) ......................................... 32
In vitro and In vivo Evaluation of (R,S)-[11C]PAQ (Paper II) ....................... 34
In vivo Metabolism of [6-O-[11C]-methyl]PD153035 in Rat (Paper III) ........ 36
(R)-[11C]PAQ in the MMTV-PyMT Tumor Model (Paper IV) ...................... 38

FUTURE DIRECTIONS .............................................................................. 41

ACKNOWLEDGEMENTS ........................................................................ 42

REPRINT PERMISSIONS ........................................................................ 44

REFERENCES ......................................................................................... 45
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>[¹⁸F]FDG</td>
<td>[2-¹⁸F]-2-fluoro-2-deoxy-D-glucose</td>
</tr>
<tr>
<td>[¹⁸F]FLT</td>
<td>3′-deoxy-3′-[¹⁸F]fluorothymidine</td>
</tr>
<tr>
<td>[¹⁸F]MISO</td>
<td>[¹⁸F]misonidazole</td>
</tr>
<tr>
<td>¹⁴C</td>
<td>carbon-11</td>
</tr>
<tr>
<td>¹³N</td>
<td>nitrogen-13</td>
</tr>
<tr>
<td>¹⁵O</td>
<td>oxygen-15</td>
</tr>
<tr>
<td>¹⁸F</td>
<td>fluorine-18</td>
</tr>
<tr>
<td>⁶⁴Cu</td>
<td>copper-64</td>
</tr>
<tr>
<td>⁶⁸Ga</td>
<td>gallium-68</td>
</tr>
<tr>
<td>⁸⁹Zr</td>
<td>zirconium-89</td>
</tr>
<tr>
<td>ABC</td>
<td>adenosine triphosphate-binding cassette</td>
</tr>
<tr>
<td>AQ</td>
<td>anilinoquinazoline</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BCRP</td>
<td>breast cancer resistance protein</td>
</tr>
<tr>
<td>Bq</td>
<td>becquerel</td>
</tr>
<tr>
<td>Ci</td>
<td>curie</td>
</tr>
<tr>
<td>CML</td>
<td>chronic myeloid leukemia</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DOTA</td>
<td>1,4,7,10-tetraazacyclododecane-1.4,7,10-tetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>FOV</td>
<td>field of view</td>
</tr>
<tr>
<td>FWHM</td>
<td>full width at half maximum</td>
</tr>
<tr>
<td>HER2</td>
<td>human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IA</td>
<td>intra-arterial</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>inhibitory concentration, 50% of maximum</td>
</tr>
<tr>
<td>IF</td>
<td>immunofluorescence</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IP</td>
<td>intra-peritoneal</td>
</tr>
<tr>
<td>IV</td>
<td>intra-venous</td>
</tr>
<tr>
<td>K₄</td>
<td>dissociation equilibrium constant</td>
</tr>
<tr>
<td>Kᵢ</td>
<td>dissociation equilibrium constant (inhibition)</td>
</tr>
<tr>
<td>LAH</td>
<td>lithium aluminum hydride</td>
</tr>
<tr>
<td>MMTV</td>
<td>mouse mammary tumor virus</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NSCLC</td>
<td>non-small cell lung cancer</td>
</tr>
<tr>
<td>PAQ</td>
<td>3-piperidinylethoxy-anilinoquinazoline</td>
</tr>
<tr>
<td>PDGFR</td>
<td>platelet derived growth factor receptor</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PI</td>
<td>phosphor imaging</td>
</tr>
<tr>
<td>PVE</td>
<td>partial volume effect</td>
</tr>
<tr>
<td>PyMT</td>
<td>polyoma middle T</td>
</tr>
<tr>
<td>RGD</td>
<td>arginylglycylaspartic acid</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>SC</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SR</td>
<td>specific radioactivity</td>
</tr>
<tr>
<td>SUV</td>
<td>standardized uptake value</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TK</td>
<td>tyrosine kinase</td>
</tr>
<tr>
<td>TKI</td>
<td>tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>
INTRODUCTION

1 IMAGING TECHNIQUES

Imaging of the body can provide detailed information about anatomical structures, physiological conditions and biological functions without the need for invasive procedures. When imaging is used in oncology, the disease can be diagnosed and characterized and changes due to progress or treatment effects can be monitored over time. This thesis has focused on the evaluation of two radiotracers and their tracing abilities and applications in positron emission tomography (PET). In two of the studies either computed tomography (CT) or magnetic resonance imaging (MRI) was used in combination with PET. The *ex vivo* imaging techniques, phosphor imaging (PI) and immunohistochemistry (IHC) are used in paper II-IV to obtain detailed information of radioactivity distribution and expressions of biomarkers, respectively, from resected tissue samples.

1.1 Positron Emission Tomography

PET is a non-invasive molecular imaging technique that allows for the study of biochemical processes and quantification of specific enzymes and receptors in the living body (*in vivo*). A tracer molecule labeled with a positron emitting radionuclide is injected into the patient or study subject. Upon decay, the radionuclide emits a positron which, after slowing down, subsequently combines with an electron within a short distance from the decay (one to a few millimeters). The two particles are annihilated and two 511 keV photons are emitted at an angle of approximately 180 degrees. The high energy photons travel out of the body and can be detected using a PET camera\(^{(1)}\) (fig. 1). When both photons are detected within a predefined time window of a few nanoseconds, the event is recorded as a coincidence. By tracking the coincidences, the location of the radioactive decay can be approximated. Data is recorded from the part of the body or subject which is located in the field of view (FOV).

After the PET scan, the large amount of recorded data is reconstructed into 3D-volumes of the distribution of radioactivity. Dynamic series of images can be created which allows observations of changes in tissue concentrations and excretion of radioactivity during the time of the scan. Quantitative images are obtained after appropriate corrections for radioactive decay, tissue attenuation, detector dead time, random events and scattered photons\(^{(2)}\).
Figure 1. Principles of PET. Decay of a radionuclide occurs with a emission of a positron which subsequently annihilates with an electron to emit two 511 keV photons that can be detected by the PET camera. The 3D-image (volume rendering technique) is reconstructed from data acquired for 60 min after injection of \((R)\)\(^{[11]}\)C]PAQ in a female wild type (WT) mouse. The image is part of the biodistribution studies conducted in paper IV.

In addition to many engineering factors, the spatial resolution of a PET camera is limited by the distance that the positron travels before it is annihilated. The distance depends on the kinetic energy of the photon, which varies for each positron-emitting radioisotope (table 1), and also on the density of the material in which the decay has occurred.

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Half-life (min)</th>
<th>Maximum Energy (MeV)</th>
<th>Mean range in water (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{18})F</td>
<td>109.7</td>
<td>0.64</td>
<td>0.64</td>
</tr>
<tr>
<td>(^{11})C</td>
<td>20.4</td>
<td>0.96</td>
<td>1.03</td>
</tr>
<tr>
<td>(^{13})N</td>
<td>9.96</td>
<td>1.19</td>
<td>1.32</td>
</tr>
<tr>
<td>(^{15})O</td>
<td>2.07</td>
<td>1.70</td>
<td>2.01</td>
</tr>
</tbody>
</table>

Table 1. Some properties of positron emitting radionuclides commonly used in PET. Half-lives, kinetic energies of the emitted positrons and their mean theoretical ranges in water are listed\(^(3)\).

The limited resolution can be an issue, especially when examining the distribution of radioactivity in small animals. The size of the body components are smaller compared to humans and thus the relative resolution is less accurate, with an increased risk of spillover effects between different tissue compartments. However, the resolution in dedicated small animal scanners is improved by the smaller gantry size, which allows for better accuracy of the coincidence determination. The resolution of a human PET-camera is generally between 4-6 mm while that of a dedicated small animal PET-camera is in the 1 mm range\(^(4)\). The camera used in the experiments presented in the papers of this thesis (II-IV) is the microPET Focus 120\(^\circledR\) (Siemens/CTI Concorde MicroSystems) which has an axial FOV of 7.6 cm and a spatial resolution of 1.3 mm\(^(5)\) (fig. 2).
1.1.1 Data Processing and Image Analysis

Data processing and analyses were performed with Siemens/CTI Concorde software. The following description and discussion applies to the setup of software and hardware used in papers II-IV. After the PET scan, the acquired listmode raw data has to be processed into a sinogram file. Files containing dynamic image data can be created in this step. In a dynamic file the data is separated into time frames, usually in the range of 1 to 600 s, which provides the opportunity to follow changes in radioactivity distribution over time and to choose a more detailed time resolution for interesting events, such as the initial distribution phase of the tracer. The created sinogram file can subsequently be reconstructed into an image file, which can be read and analyzed in various programs. When creating the image file, reconstruction corrections can be applied for attenuation (tissue stopping the photons before they leave the body), and dead time (detectors being saturated by large numbers of photons emitted in the FOV). Attenuation correction was not applied in any of the studies in this thesis. When using mice or studying superficial structures in rat, e.g. subcutaneous (SC) tumors, the impact of attenuation is generally considered negligible due to the small amount of tissue the photons have to penetrate before they leave the body. However, in the rat biodistribution studies in papers III and IV attenuation correction would have been preferred. Corrections algorithms are usually also applied for scatter and random coincidences, both contributing to increased noise levels in the images. Scatter is when a photon collides with an electron and changes direction before it hits the detector. Random coincidences are registered when photons from two individual events are detected within a predefined time window, and therefore mistakenly registered as one single event.

Other phenomena compromising the quantitative imaging of small structures and delineation of tissues, that sometimes should be considered are the partial volume effects.
(PVE) and spillover effects - both caused by the finite spatial resolution of PET. The PVE occurs when a structure is smaller than approximately three times the full-width at half maximum (FWHM) value and causes the uptake of radioactivity in the structure to be less than the real value and that in the surrounding tissue to be greater than the actual\(^6,^7\). FWHM is a hardware and isotope specific measurement of the spatial resolution, usually performed with fluorine-18\(^{(18}F)\) in the center of FOV. For the microPET Focus 120® camera the FWHM is 1.3 mm\(^5\), although it would be larger for carbon-11\(^{(11}C)\) due its higher position energy (table 1). An example where PVE can cause problems is quantification of uptake in small organs such as the adrenal glands in rodents. In paper IV it was virtually impossible to distinguish the \((R)-N-(4-bromo-2-fluorophenyl)-6-methoxy-7-(1-{^{11}}C-methyl-3-piperidinyl) methoxy\) -4-quinazolinamine \((R)-[{^{11}}C]PAQ\) uptake in the mouse adrenal glands, < 2 mm in diameter, from the kidney, though it was clearly distinguishable in rat adrenal glands with a diameter of ~5 mm. Another example is the small, ~1 mm in diameter, pulmonary metastases detected in paper IV. The spillover effect is essentially caused by the same physical problem but, as opposed to the PVE, areas adjacent to tissues with high uptake will gain in activity. The spillover effect problems can be exemplified with the \((R)-[{^{11}}C]PAQ\) distribution in paper IV where the liver uptake was very high and spills over to the adjacent parts of the lungs. In these images, the parts of the lungs in close proximity to the liver were therefore deliberately not included in the regions of interests (ROIs).

In the tumor uptake quantification studies in papers II-IV, 3D-ROIs were placed over the tumor areas. In some cases it was not possible or very uncertain to delineate the tumors based on radioactivity uptake after administration of \([6-O-{^{11}}C-methyl] 4-N-(3-bromoanilino)-6, 7-dimethoxyquinazoline \(([^{11}]C)PD153035) or \([^{11}]C]PAQ\). In a few experiments, described in papers II and IV, \([2-{^{18}}F]2-Fluoro-2-deoxy-D-glucose \(([^{18}]F]FDG)\) was injected after the scan with \([^{11}]C]PAQ\) and served as a marker of tumor volume and location. In papers III and IV, CT and MRI were used to acquire morphological information of the tumors which was subsequently used for mapping the PET uptakes. Software tools were used to align the images from the different modalities. Specific organ uptakes were used as landmarks during the alignment, e.g. heart and bladder for \([^{18}]F]FDG/MRI and liver and kidneys for \([^{11}]C]PAQ/MRI in paper IV. In cases where neither \([^{18}]F]FDG PET data nor any morphological information was acquired, ROIs were placed over the tumor regions based on postmortem measurements of tumor dimensions and uptake in the PET images, although this method is more uncertain, especially when uptake is low and/or heterogeneous.

1.2 Computed Tomography

CT is based on the variable absorption of X-rays that are emitted from a source in the camera. On the opposite side, the X-rays are registered by detectors and the collected data is reconstructed into 2D or 3D images of the subject. Thus the morphology of the subject based on tissue density is depicted (fig. 3).
Figure 3. CT imaging: X-rays pass through the subject to varying degrees depending on the density of the material through which they pass and the acquired data is reconstructed into images. The image to the right is the upper part of a rat, tumors (red arrows) and an intra-arterial catheter (yellow arrow) are visible in the image. Further explanation of the image and experimental setup can be found in paper III.

In oncology, CT is most often used alone for detecting tumors and following changes in their sizes over time, but also increasingly in combinations with PET (see section 1.4) to improve the morphological accuracy of the functional information obtained. Low dose CT is currently tested in screening for early stage lung cancer in high risk groups, e.g. smokers. The screening has been shown to reduce mortality due to the chance for earlier treatment.(8, 9).

Another application is CT angiography, the imaging of blood vessels that have been opacified by a contrast medium. The method is used in diagnostic cardiovascular examinations and as a tool in interventional procedures for locating and removing obstructions in the vasculature.(10). In one of the experiments in paper III, CT was used for positioning an intra-arterial (IA) catheter and angiography was used to verify the blood flow from the catheter to the tumor area before injection of the tracer.

1.3 Magnetic Resonance Imaging

MRI is an imaging modality which is primarily used to obtain morphological images based on the nuclear magnetic resonance (NMR) signal from the hydrogen atoms in molecules in a patient or object. The NMR signal is acquired by placing the subject in a static magnetic field and then applying a pulsating radio-frequency magnetic field that changes the spin direction of the hydrogen atoms. The NMR signal is created upon spin relaxation and is recorded by coils in the camera. A higher concentration of hydrogen
gives a stronger signal. The acquired information is reconstructed into 2D-images or 3D volumes of the object\(^{(11)}\) (fig. 4).

**Figure 4.** Principles of MRI. The magnetic fields inside the camera give rise to NMR signals. I: the spin is aligned to the magnetic field in the camera, II: a second magnetic pulse is applied and the spin direction is changed, III: the second magnetic pulse is discontinued and the subsequent spin relaxation generates an NMR signal that can be registered and reconstructed into images. The 2D-coronal image to the right is from paper IV and shows a euthanized female mouse carrying mammary gland tumors (red arrows).

NMR signals from water and fat, the major hydrogen containing components of living organisms, are examined when used in medical imaging. By changing certain camera parameters when acquiring the signal, different information can be obtained and specific protocols can be used based on type of examination and location of the area of interest. In clinical oncology, MRI is used in the diagnosis and treatment monitoring of e.g. breast cancer, prostate cancer and brain tumors and has also been evaluated as an complimentary screening platform for breast cancer\(^{(12-14)}\).

### 1.4 Combining Modalities

Multimodality imaging means that the information obtained from at least two different imaging modalities are combined. The additional information often provides synergistic accuracy in diagnosis and in research. When combining PET with either MRI or CT the biochemical and functional information provided by PET can be mapped over the morphological information provided by CT or MRI. Multimodality imaging is used in both paper III and paper IV, although the cameras used here were separate units.

Combined/hybrid PET/CT cameras are now standard clinical modalities, greatly increasing the diagnostic value by allowing for a more precise discrimination between physiologic
and malignant uptake of PET-tracers\(^{(15, 16)}\). The combination is routinely used in staging various cancers and in planning tumor tissue resection procedures\(^{(17-19)}\). In preclinical imaging both PET/CT and PET/MRI cameras show promising results and have the potential to greatly increase the impact of small animal imaging\(^{(20, 21)}\).

### 1.5 Ex vivo Immunohistochemistry

IHC is a technique used to find and sometimes quantify specific proteins or structures in tissues at a microscopic level. In order to perform a standard IHC procedure, the resected tissue is frozen and cut into thin sections using a cryostat. The sections are subsequently fixed before applying the primary antibodies. Common methods involve fixations of tissue preparations with formalin and paraffin embedding. Primary antibodies that bind to the structure of interest are incubated with a tissue section using specially designed protocols. Usually subsequent additions of secondary antibodies are used to allow for staining and visualization of the binding sites of the primary antibodies. Secondary antibodies can be conjugated to an enzyme, such as peroxidase (immunoperoxidase staining). Alternatively, the secondary antibody can be tagged to a fluorophore, such as fluorescein or rhodamine, which re-emits light upon light excitation (immunofluorescence (IF)). In IF, several structures can be studied by sequential addition of different primary and secondary antibodies\(^{(22)}\). IHC and IF can be used to diagnose and characterize isolated tissues, e.g. histopathological analyses of tumors. Traditional IHC with immunoperoxidase staining is used in paper II and III for estimating the presence of RTKs in tumor tissue while multiplex IF is used in paper IV for a characterization of primary tumors and lung tissue with suspected metastatic activity (fig. 5).

**Figure 5.** IHC of mouse lung tissue. To the left (I), immunoperoxidase staining of the VEGFR2 expression (brown color) and hematoxylin staining of the cell nuclei (blue). To the right (II), an IF image in approximately the same magnification (20X), where 4',6-diamidino-2-phenylindole (DAPI) staining (blue) indicates cell nuclei and VEGFR2 staining is indicated by the red fluorescence. The round structure in the middle of the image is a blood vessel with a diameter of approximately 200 µm. Details on the two techniques are described in papers II and IV.

The methods are often referred to as semi-quantitative since limitations are often present. Cross-reactivity between antibodies has to be considered as well as saturation issues and
difficulties in standardizing the staining protocols\textsuperscript{(23)}. Often a limited amount of tissue is analyzed from a sample which can cause biases when extrapolating results to a larger area or to e.g. several tumors since the biomarkers may be heterogeneously distributed.

\subsection*{1.6 Ex vivo Phosphor imaging}

PI is widely used in connection with preclinical PET imaging as a method for studying tracer distribution \textit{ex vivo} with increased resolution\textsuperscript{(24)}. The technique also offers an opportunity for correlating the radioactivity distribution to other \textit{ex vivo} analyses\textsuperscript{(25, 26)}, e.g. IHC or IF. It is a quantitative technique in which ionizing radiation from thin slices (10-30 \textmu m) of tissue is exposed to phosphor screens which are subsequently read in a plate reader. The radiation from the decaying radionuclides in the tissue section excites atoms on the plate and upon release of the stored energy, usually with a laser-induced stimulation, the information can be read and stored digitally. The sensitivity of the technique is generally better than with ordinary X-ray based autoradiography\textsuperscript{(27)} and is suitable for short-lived PET-radionuclides such as \textsuperscript{11}C and \textsuperscript{18}F. The method is used in paper II and IV to correlate the uptake of radioactivity to biomarkers present in the tissue.

\section*{2 PET RADIOTRACERS}

The most commonly used positron emitting radionuclides, \textsuperscript{11}C and \textsuperscript{18}F can potentially be incorporated in almost any known biologically active molecule by using the appropriate radiochemical strategies for labeling. By replacing one of the atoms present in the molecule with the corresponding radioisotope a positron emitting analog is produced, possessing otherwise identical biological and chemical properties. The two radiotracers described in this thesis are based on molecules belonging to a group of drugs called tyrosine kinase inhibitors (TKIs). Other TKIs evaluated for the use in PET are described, as well as radiotracers with similar diagnostic applications. A few other tracers, commonly used in clinical oncology, are described in section 4.4.

\subsection*{2.1 Tyrosine Kinase Inhibitors}

Several radiotracers based on TKIs have been evaluated for \textit{in vivo} imaging (fig. 6). Of the clinically used 4-anilinoquinazolines (4-AQ) described in table 3 (section 4.5), vandetanib\textsuperscript{(28)}, erlotinib\textsuperscript{(29)}, gefitinib\textsuperscript{(30)} and lapatinib\textsuperscript{(31)} have all been labeled with \textsuperscript{11}C or \textsuperscript{18}F and all but \textsuperscript{[11}C]vandetanib evaluated in either preclinical or clinical imaging pilot studies. \textsuperscript{11}C-Labeled lapatinib is currently being evaluated as a predictor of drug uptake in human epidermal growth factor receptor 2 (HER2)-positive breast cancer patients with brain metastases. Initial results are promising with tumor tissue uptake being higher compared to normal tissue\textsuperscript{(32)}.\n

Preclinical studies with the epidermal growth factor receptor (EGFR) targeting tracer $[^{11}\text{C}]$gefitinib showed promising results with indications of good receptor quantifying abilities\(^{(33)}\). However, in another study with the $^{18}\text{F}$-labeled analog, it was concluded that high non-specific binding compromises quantification and that the tracer is better suited for predicting gefitinib pharmacokinetics and tumor uptake based on other mechanisms than receptor specific binding\(^{(34)}\).

Initial preclinical studies with EGFR targeting $[^{11}\text{C}]$erlotinib revealed that the tracer has the ability to predict response to EGFR targeted therapy\(^{(29)}\). The tracer has since been further evaluated with rather positive results in clinical pilot studies. In a case report of a non-small cell lung cancer (NSCLC) patient with brain metastasis, the tracer was able to visualize the metastatic tissue. The patient carried an erlotinib-sensitizing exon 19 deletion mutation in the primary tumor and responded well to post-imaging erlotinib treatment, as evaluated with MRI and CT\(^{(35)}\). In another study with 13 NSCLC patients the tracer was able to predict therapy response\(^{(36)}\). All patients in which pre-treatment $[^{11}\text{C}]$erlotinib PET showed marked hotspots in the lung area, responded to erlotinib treatment while two of six patients without noticeable uptake of $[^{11}\text{C}]$erlotinib responded. The authors emphasize the importance of correct characterization of the tumors since differences in EGFR expression were observed between tumors in the same patients. This could easily be missed by conventional biopsies while the results in this study indicated that $[^{11}\text{C}]$erlotinib PET could more correctly characterize specific lesions. In addition the tracer was able to visualize tumor tissue that was not detectable with $^{18}\text{F}$FDG. Further studies with $[^{11}\text{C}]$erlotinib on NSCLC-patients with and without the exon 19 deletion mutation showed that the tracer was taken up in significantly higher levels in lesions carrying the mutation and thus able to predict therapy.

**Figure 6.** The 4-AQ core structure and some clinically used TKIs that have been labeled with positron-emitting radionuclides.
response in this patient group\textsuperscript{(37)}. In this study the uptake of tracer was, however, not correlated with EGFR expression levels, nor with tumor blood flow assessed with \textsuperscript{[\textsuperscript{15}O]}H\textsubscript{2}O PET. The apparent success in determining mutational status of NSCLC patients with \textsuperscript{[\textsuperscript{11}C]}erlotinib may be explained by changes in erlotinib binding affinity conferred by the exon 19 deletions. A $K_i$ value of 3.3 nM, compared to 17.5 nM, in WT receptors has been reported\textsuperscript{(38)}.

Additional studies have been performed by a few groups on a whole series of irreversible binding TKIs with the 4-AQ core structure\textsuperscript{(39-46)}. The molecules having good \textit{in vitro} characteristics with $K_i$ values mostly in the µM range have been at most modestly capable of targeted \textit{in vivo} imaging. The poor results have been shown or speculated to depend on high non-specific binding, a fast tissue clearance, and low \textit{in vivo} metabolic stability. The metabolism was however not studied for most of these molecules.

TKIs with chemical structures other than 4-AQ that are used clinically and have been labeled and evaluated for PET-studies include the multi receptor tyrosine kinase (RTK) targeting drugs imatinib\textsuperscript{(47)} and sorafenib\textsuperscript{(48, 49)}. The study on \textsuperscript{[\textsuperscript{11}C]}imatinib was performed to evaluate the distribution of non-labeled drug in normal tissue. In one study on \textsuperscript{[\textsuperscript{11}C]}sorafenib, the tracer was evaluated in preclinical tumor models with rather promising results\textsuperscript{(48)} showing good tumor uptake. However, the multi targeting properties of the molecule make the results difficult to interpret. The other study on \textsuperscript{[\textsuperscript{11}C]}sorafenib evaluated the impact on brain uptake by efflux proteins\textsuperscript{(49)}.

The binding of TKIs to efflux proteins and the implications on their distributions are further discussed section 3.2.2.

\textbf{2.1.1 \textsuperscript{[\textsuperscript{11}C]}PD153035 and Tracer Analogs}

![Figure 7. [6-O-\textsuperscript{11}C-methyl]PD1530351 and [7-O-\textsuperscript{11}C-methyl]PD153035.](image)

PD153035 is a reversibly binding 4-AQ TKI targeting the EGFR. The \textit{in vitro} properties are excellent with a $K_i$ of 5.2 pM and an $IC_{50}$ value of only 29 pM\textsuperscript{(50, 51)} against the EGFR. These characteristics made the molecule a highly interesting candidate as a PET tracer and has led to several studies evaluating the \textsuperscript{11}C-labeled tracer and its analogs as EGFR imaging probes. \textsuperscript{[\textsuperscript{11}C]}PD153035 was first labeled in\textsuperscript{(52)} and\textsuperscript{(53)} by methylation of the $O$-desmethyl group in either position 6 or 7 (fig. 7). The tracer has been evaluated in several preclinical studies; the first in rats with and without neuroblastoma implants\textsuperscript{(54)}. In that study, the tumor uptake was rather low with a maximum level of approximately 0.3% of the injected dose/g. However, lacking IHC confirmation of EGFR expression levels, it is difficult to characterize the nature of the tumor uptake. Further preclinical studies in mice with
different SC tumor implants showed a correlation between EGFR expression and tracer uptake\(^{(55, 56)}\). A specific binding to the EGFR was also demonstrated by pretreatment blocking with unlabeled PD153035\(^{(56)}\).

In a human pilot study, biodistribution and dosimetry of \[^{11}C\]PD153035, was evaluated. High concentrations of radioactivity were found in liver, gallbladder and kidneys - organs with excretory functions. The excreted amounts of radioactivity were very high, with 25-40% of the injected dose found in the GI tract or urinary bladder 60 min after injection. The main route of excretion was via the kidneys to the urinary bladder, which also received the highest dose of radioactivity. It was concluded here that radioactivity uptake did not correlate to EGFR expression levels in peripheral organs which, according to the authors, might depend on the radioactivity in these tissues being mostly in the form of radioactive metabolites\(^{(57)}\).

Another study on tumor uptake of \[^{11}C\]PD153035 in patients with chemotherapy-refractory NSCLC reported a correlation between uptake of radioactivity and effectiveness of EGFR directed therapy with the TKI erlotinib. The maximum standardized uptake values \((SUV_{\text{max}})\) were used here to assess the tumor uptake of \[^{11}C\]PD153035. Measurements started 20 min after radiotracer injection, when tissue levels had stabilized. The metabolism and contribution of radiometabolites on tumor uptake was not considered in this study, nor were the EGFR expression levels or mutational status of the patients confirmed and correlated to uptake levels\(^{(58)}\). The uptake in tumors did not change after initiation of erlotinib treatment, though a displacement effect at the EGFR could have been expected if tracer uptake reflected specific EGFR-binding. As in the dosimetry study\(^{(57)}\), the labeling position of \[^{11}C\]PD153035 was not stated in this paper.

Among the PD153035 derivatives that have been labeled with positron-emitting radionuclides, a series of molecules labeled with \(^{18}F\) in the aniline-ring were evaluated \textit{in vivo} in mice. Due to a suspected significant metabolism and unfavorable biodistribution the substances were deemed unsuitable for \textit{in vivo} imaging according to the authors\(^{(59, 60)}\). These results with the derivatives are analogous to ours in paper III with \[^{11}C\]PD153035: that extensive \textit{in vivo} metabolism most likely impairs capability for EGFR-imaging.

2.1.2 \[^{11}C\]PAQ and Tracer Analogs

PAQ is an acronym for 3-piperidinylethoxy-anilinoquinazoline – the core structure of this 4-AQ. The \((R)-N-(4-bromo-2-fluorophenyl)-6-methoxy-7-((1-methyl-3-piperidinyl)-methoxy)-4-quinazolinamine ((R)-PAQ)\) (fig. 8) was the molecule with the highest reported affinity for the VEGFR2 in a series of 4-AQs with basic side chains in position 7. The molecules were synthesized and evaluated \textit{in vitro}\(^{(61)}\). The \(S\)- and \(R\)-stereoisomers were reported to have \(IC_{50}\)-values of 10 and 1 nM, respectively, for the VEGFR2 at competitive concentrations of 2 \(\mu\)M adenosine triphosphate (ATP). The affinity for other RTKs was also investigated with the lowest reported \(IC_{50}\) values determined for the EGFR: 0.1 \(\mu\)M for the \(S\)-isomer and 0.2 \(\mu\)M for the \(R\)-isomer. This means that the specific binding,
regarding RTKs, for VEGFR2 is likely better for the R-isomer with a 200-fold higher affinity for the VEGFR2 versus EGFR compared to only a 10-fold difference for the S-isomer. This data and the reported 10-fold higher affinity for the VEGFR2 convinced us that further studies with $[^{11}\text{C}]\text{PAQ}$ was better performed with the pure R-isomer (paper IV) after the initial promising studies with the racemate (paper II). The results with $(R,S)$-$[^{11}\text{C}]\text{PAQ}$ and $(R)$-$[^{11}\text{C}]\text{PAQ}$ are described and discussed for each paper in the Results and Comments section.

Three other molecules structurally similar to $(R,S)$-$[^{11}\text{C}]\text{PAQ}$ have been developed for potential applications in PET. The non-chiral $[^{11}\text{C}]\text{vandetanib}$ (fig.8) and $[^{11}\text{C}]\text{chloro-vandetanib}$ have been successfully labeled but not yet evaluated in vivo$^{(62)}$. $(R)$-$[^{18}\text{F}]\text{FEPAQ}$, a fluoroethyl analog of $(R)$-$[^{11}\text{C}]\text{PAQ}$, has shown promising results in ex vivo binding assays in tissue from a resected human glioblastoma tumor$^{(63)}$.

### 2.2 Antibodies, Proteins and Peptides

Other approaches in PET quantification and visualization of RTKs and associated receptor ligands include using labeled monoclonal antibodies such as cetuximab and bevacizumab. Cetuximab is used in the treatment of metastasized colorectal cancer and squamous cell carcinomas of the head and neck$^{(64)}$. The antibody acts as an antagonist and binds to the extracellular ligand binding domain of the EGFR and is thus a candidate ligand for quantification of EGFR. Cetuximab has been labeled with the positron emitting-radionuclides copper-64 ($^{64}\text{Cu}$) and zirconium-89 ($^{89}\text{Zr}$) via a 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-chelator. In preclinical studies with xenograft bearing mice, the uptake of the $^{64}\text{Cu}$ labeled antibody correlated well with the EGFR expression found in tumor tissue$^{(65)}$. However, in the study with $[^{89}\text{Zr}]\text{cetuximab}$ the uptake was highest in tumors expressing intermediate levels of EGFR and no correlation between uptake and receptor expression was found$^{(66)}$.

Bevacizumab binds to and immobilizes extracellular vascular endothelial growth factor (VEGF)-A and thus decreases vascular endothelial growth factor receptor 2 (VEGFR2)
signal transmission (see section 4.3.2). It is therefore used as an anti-angiogenic drug in treatment of several cancer types including HER2 negative breast cancer, colorectal cancer and NSCLC\(^{(64)}\). Bevacizumab labeled with \(^{89}\)Zr has been shown to have higher uptake in angiogenic tumor tissue compared to levels of non-specific binding of \(^{89}\)Zr-IgG\(^{(67)}\). In another study, \(^{89}\)Zr-bevacizumab uptake levels were correlated to angiogenic activity during treatment and to the efficacy of the antitumor drug\(^{(68)}\).

Interesting imaging results have also been reported with Affibody molecules. These molecules are relatively small (usually 58 amino acid residues) and highly stable scaffold proteins. The molecules binds to the extracellular ligand binding domain of RTKs. EGFR targeting with PET has been performed with the Affibody \(^{18}\)F-FBEM-Cys-Z\(_{EGRF:1907}\). In studies with tumor xenograft-bearing mice, the results indicate that the tracer is able to differentiate between low and high levels of EGFR in the tumors. Tumor imaging was improved by pretreatment with unlabeled substance, blocking non-tumor binding sites, at doses low enough to not displace the labeled molecule from binding to EGFR in the tumor\(^{(69)}\). Similar results were observed with the same Affibody labeled with \(^{64}\)Cu, via a DOTA-chelator, in a hepatocellular carcinoma xenograft model\(^{(70)}\). Another promising Affibody for RTK imaging is the HER2 targeting Z\(_{HER2:342}\). The molecule has been labeled with \(^{11}\)C and gallium-68 (\(^{68}\)Ga) via a selenium-tag and binds with high affinity and specificity to HER2 in tumor xenografts\(^{(71)}\). Further studies with \(^{18}\)FZ\(_{HER2:342}\) reported equally promising results and also advantages over \(^{18}\)FFDG regarding differentiation between uptake in inflammatory tissue and HER2 expressing tumors\(^{(72)}\).

In addition to the previously described tracers, labeled peptides based on the Arg-Gly-Asp (RGD) amino acid sequence have been evaluated as imaging probes for angiogenesis. The small peptides bind with high affinity to the integrin alpha(v)beta3 which is expressed during angiogenesis. The RGD based PET-tracers have been shown to be able to rather accurately estimate levels of in vivo angiogenesis. The current research has been reviewed in\(^{(73)}\). One of the more studied RGD-peptides is \(^{18}\)FAH111585, which is currently evaluated in clinical trials for angiogenesis quantification and tumor detection\(^{(64)}\).

### 2.3 Radiolabeling with Carbon-11

The radiolabeling and isolation of PET tracers need to be performed as quickly as possible, with the time between the start of synthesis and administration to the patient or animal ranging from a few minutes to a few hours depending on the radionuclide used. In the papers of this thesis the two radiotracers have both been labeled with \(^{11}\)C by alkylation reactions with \(^{11}\)C-methyl iodide (\(^{11}\)CCH\(_3\)I). The methylations were performed on the O-desmethyl or N-desmethyl precursors of \(^{11}\)C-PD153035 and \(^{11}\)C-PAQ respectively.

In papers I and II the “wet” method for producing \(^{11}\)CCH\(_3\)I was used, which includes the cyclotron (PETtrace; GE Healthcare AB) production of \(^{11}\)CCO\(_2\), conversion to \(^{11}\)CCH\(_3\)OH via reduction with lithium aluminum hydride (LiAlH\(_4\)) in tetrahydrofuran (THF) followed by reaction with hydroiodic acid (HI) to give \(^{11}\)CCH\(_3\)I (fig. 9). This
precursor was first introduced in the 1970’s\(^{(74, 75)}\) and further improved by Johnström et al.\(^{(76)}\), who developed a one-pot synthesis. The precursor has since been widely used in \(^{11}\)C mono-alkylation reactions, although this production method generally gives a somewhat lower specific radioactivity (SR) since naturally occurring unlabeled CO\(_2\) is mixed into the initial conversion steps\(^{(77)}\).

The other method for producing \([^{11}\text{C}]\text{CH}_3\text{I}\) used in paper III and IV, is performed by the conversion of cyclotron-produced \([^{11}\text{C}]\text{CH}_4\) by gas phase reaction with iodine as described in\(^{(78)}\) (fig. 9). The method often gives a much higher SR when the methods are based on CH\(_4\) instead of CO\(_2\) since dilution from naturally occurring sources of CH\(_4\) is much lower. An automated synthesis module (Tracerlab FX C Pro; GE Healthcare AB) was used for the radiolabeling and purification of the tracers in these papers.

![Figure 9. Conversion of \([^{11}\text{C}]\text{CO}_2\) (I) and \([^{11}\text{C}]\text{CH}_4\) (II) to reactive \([^{11}\text{C}]\text{CH}_3\text{I}\).](image)

General radiolabeling procedures for the tracer productions in all studies started with the reaction of 0.7-1.0 mg of the desmethyl-precursor with the trapped \([^{11}\text{C}]\text{CH}_3\text{I}\) in DMF with approximately 10 mg of K\(_2\)CO\(_3\) added. Reactions were carried out at 95°C for 5 min before the tracer was purified by semi-preparative HPLC and solid phase extraction with 99.5% ethanol. Details on the radiosynthesis and purification procedures are given in the individual papers.

### 2.3.1 Specific Radioactivity

SR provides a measure of the amount of labeled molecules (Bq or Ci) in relation to the total amount (mol) of the substance in the same chemical form. For PET tracers the SR is usually given as $\text{GBq}/\mu\text{mol}$ or $\text{Ci}/\mu\text{mol}$. As the radionuclides decay, the SR will decrease. When expression levels of the intended target are low, the SR becomes very important since the competition with unlabeled molecules for binding sites will increase as well as the risk of saturating the binding sites of the biological system, when administering the same amount of radioactivity. When the radiotracer is displaced by non-labeled substance, the quantitative estimations of the number of binding sites becomes less reliable and can be underestimated when based on radioactivity uptake\(^{(79, 80)}\).

The SRs for most of the experiments in papers III and IV were found to generally be 500-1000 GBq/µmol at injection. When using the wet method to produce the labeling precursor in papers I and II, values were considerably lower with a SR in the order of 30 GBq/µmol. These differences in SR may have, to some degree, affected the imaging and metabolism results. In paper I when the metabolism of \([^{11}\text{C}]\text{PD153035}\) was studied, a higher SR might
have yielded more radiometabolites at a higher rate but the general trends in metabolism were most likely not affected. Accordingly, in paper III, a lower SR of $[^{11}\text{C}]$PD153035 might have decreased the formation rate of radiometabolites in vivo if concentrations that saturated CYP-enzyme activity had been reached. In the studies of $[^{11}\text{C}]$PAQ, the differences in SR between paper II and IV could possibly have affected the tumor imaging abilities. However, since different stereoisomer compositions were used and the disease models were rather different, isolation of the impact of different SRs is difficult.

3 PHARMACOKINETICS

Pharmacokinetic studies aim to reveal the fate of a drug after the administration to a living organism and include studies on absorption, distribution, metabolism and excretion. In PET studies, the radiotracer is often administered intra-venously (IV) and thus the absorption phase is not applicable. When evaluating new radiotracers it is important to examine their pharmacokinetic behavior before moving on to possible disease characterizations or receptor mapping experiments to minimize risks of misinterpreting results.

3.1 Metabolism In vitro and In vivo

Experiments with the purpose of assessing the metabolism of the two radiotracers evaluated in this thesis were performed in all four papers. In papers I and II we examined the in vitro metabolism of $[^{11}\text{C}]$PD153035 and $[^{11}\text{C}]$PAQ, respectively, and in papers III and IV their metabolism in vivo.

When using radiolabeled tracers it is very important to remember that the in vivo PET images describe the distribution of radioactivity and not necessarily that of the labeled tracer. Depending on the structural characteristics of the tracer and the metabolic properties of the subject being examined, metabolism might transform the tracer to other molecules carrying the radionuclide. A considerable metabolism during the scanning time will subsequently yield images not only showing the actual distribution of the intended target but also the distribution of the metabolites and their potential targets. Data obtained can thus be misinterpreted if the metabolism is not taken into consideration and models for metabolite correction are not applied.$^{81,82}$

Metabolism, or biotransformation, generally produces metabolites that are more hydrophilic than the parent compound and can therefore be more readily excreted from the body. Metabolism can be divided into two phases, where the reactions of each phase are carried out by a unique set of enzymes. The phase I reactions remove or insert groups in the molecule and expose them for subsequent conjugating phase II metabolism. Phase I reactions are not always necessary prior to phase II reactions. Phase I reactions include hydrolysis, oxidation and reduction reactions while phase II reactions are often conjugations, e.g. acetylation or glucuronidation. Phase I reactions are mainly conducted by cytochrome P450 (CYP) enzymes located in the microsomes in the endoplasmic
reticulum of liver cells. Phase II conjugation reactions are performed by transferase enzymes located in the liver and in extra-hepatic tissue like the kidneys and intestine\(^{(83)}\).

Isolated microsomes are used in many \textit{in vitro} metabolism assays to predict \textit{in vivo} metabolism. The microsomes contain many of the phase I metabolizing CYP enzymes\(^{(84)}\). Oxygen and energy in the form of nicotinamide adenine dinucleotide phosphate (NADPH) have been shown to be essential for the function of the enzymes\(^{(85)}\). Although the \textit{in vitro} assays can have some predictive value for \textit{in vivo} metabolism, the results can be misleading when e.g. the substrate inhibits the metabolizing enzymes, which will cause underestimation of metabolic rate. In addition, some phase II reactions will not occur in microsomal preparations since the responsible enzymes or reactants are missing\(^{(86)}\). Other reports show that the interplay between efflux transporters such as P-glycoprotein (P-gp) and metabolizing enzymes can play an import role for the metabolism of compounds that are also substrates for transporters expressed in hepatic cells\(^{(87)}\).

\subsection*{3.1.2 Metabolism of Tyrosine Kinase Inhibitors}

Small molecule TKIs are mainly metabolized by CYP3A4 in humans while other CYP enzymes such as CYP2D6 and CYP1A2 play a secondary role\(^{(88)}\), see also table 3 in section 4.5. In 4-AQ-based TKIs, the phase I oxidation reactions often lead to N-oxidation in the quinazoline group and dealkylation of nitrogen- and oxygen-containing groups in positions 6 and 7, if applicable\(^{(89-91)}\). Different substitutions on the quinazoline core affect how the molecule is metabolized and to which extent\(^{(92)}\). For example, the PD153035 molecule (fig. 7) and structurally similar derivatives are prone to demethylation of the methoxy groups in positions 6 and 7 as well as N-oxidation while other 6,7-disubstituted 4-AQs such as vandetanib, erlotinib and gefitinib with bulkier substitution groups in one or both of those positions (fig. 6 and 8) are metabolized to a lesser extent, with reported half-lives of 120 h\(^{(93)}\), 36 h\(^{(94)}\) and 50 h\(^{(95)}\) in man, respectively.

Phase II dehalogenations of the aniline ring in 4-AQs have been described, enzymatic dehalogenations most likely to occur in position 4 for fluoro-substituted molecules while compounds with halogens in position 2 or 3 were more metabolically stable\(^{(96)}\). The two tracers described in this thesis are halogenated as 4-bromo-2-fluorophenyl ([\(^{11}\)C]PAQ) and 3-bromophenyl ([\(^{11}\)C]PD153035) (fig. 7 and 8, respectively). Based on the literature on TKI metabolism it is possible that the studied compounds are dehalogenated to some degree, but it is likely not one of the main metabolic routes\(^{(92)}\).

In paper III the CYP-enzyme inhibitors ketoconazole and quinidine were used to investigate the impact of specific CYP-enzymes on the metabolism of \([^{11}\text{C}]\text{PD153035}\). By administering the inhibitors prior to the PET tracers, their effects can be assessed by analyzing radioactive metabolites in blood samples. In the \textit{in vivo} metabolism assays performed in paper III and IV arterial blood was sampled. Arterial compared to venous blood sampling gives a more reliable estimation of the radioactive components being distributed throughout the body. The sample is taken after the blood has passed the liver
and before the possible metabolite pattern is compromised by differential tissue distributions of the separate components. In kinetic PET-modeling an arterial input function is often used, which requires arterial blood sampling and subsequent analysis for a correct adjustment of the input function. However, models for estimation of specific tracer uptake that instead use a reference tissue have been developed\(^{(97, 98)}\). In paper III, the uptake ratio between tumor and muscle reference tissue was measured after different administration routes of the tracer, and with different compositions of tracer and radiometabolites in the blood.

The anti-fungal drug ketoconazole is a potent inhibitor of CYP3A enzymes in both human and rat and is routinely used to assess their contributions to metabolism in vitro and in vivo\(^{(99)}\). In several studies, administration of ketoconazole has been shown to elevate plasma levels of different TKIs significantly in humans\(^{(92)}\). Quinidine is a prototypical inhibitor of the CYP2D sub-family of CYP-enzymes\(^{(100)}\). CYP2D6 is the only known isoform in humans, whereas six corresponding isoforms of the CYP2D enzymes have been identified in rats (CYP2D1 through CYP2D5 and CYP2D18)\(^{(101)}\). Both CYP3A and CYP2D are known to generate the N-oxide and the radiometabolites produced by demethylation\(^{(100)}\) observed in the studies on \([^{11}C]PD153035\) in vitro and in vivo metabolism.

### 3.2 Biodistribution

When evaluating new radioligands or using established radioligands in new species, a biodistribution study is preferably performed at an early stage. A dynamic whole body biodistribution PET scan in rodents answers a lot of questions regarding the pharmacokinetic behavior of the radioligand, whether it passes important biological barriers such as the blood brain barrier (BBB), the rate and routes of excretion, and possible dosimetric challenges.

#### 3.2.1 Binding Properties

For PET imaging of the brain, e.g. of dopamine or serotonin receptors, kinetic models have been developed and factors influencing the behavior and properties of a radioligand are well studied\(^{(102)}\). On the other hand, imaging of peripheral tissues and especially tumors poses other challenges. For example, the BBB can act as a filter for radiometabolites with low lipophilicity while these have to be considered to a higher degree in peripheral tissue. Other concerns when studying tumor lesions are related to the often chaotic vascularization which can cause both bleeding into the tumor, resulting in high non-specific uptake and on the opposite, areas with low perfusion leading to a possible underestimation of target distribution. In addition, inter- and intratumoral heterogeneity of cell function and protein expression\(^{(103)}\) can further complicate quantification of tracer uptake in tumors. Since all tumors are uniquely different, the application of solid kinetic models for target quantifications are hard to implement. However, a few important terms in radiopharmacology used in the thesis are described in this section.
**Selectivity:** This term describes the degree a molecule binds to the target in relation to other possible binding sites. For a substance intended to induce a biological effect this property might not be very important, providing the off-target binding does not cause serious adverse effects. In PET, selectivity is quite important, since a large amount of non-target binding will make it difficult to interpret selective interactions in a quantitative fashion. Preferably the tracer molecule should only bind to the intended target.

**Specificity:** In pharmacology, specificity often describes how much of an effect is due to one specific action of the drug. In diagnostic imaging, specificity can be used to describe how much of the detected radioactivity that is represented by the radioligand binding to a specific target. Thus specificity and selectivity are sometimes used interchangeably in diagnostic radiopharmacology.

**Affinity** describes how tightly a ligand binds to its target e.g. receptor or enzyme. In pharmacology it is often mathematically described by the equilibrium dissociation constant $K_d$:

$$K_d = \frac{[L][R]}{[LR]}$$

where $[L]$ and $[R]$ are the concentrations of the ligand and unoccupied binding sites, respectively, $[LR]$ is the concentration of the complex formed between the two. Furthermore, $K_i$ refers to the $K_d$ of a ligand determined with inhibitory studies, typically by displacing a radioligand with the ligand of interest.

Another term describing the ability of a ligand to bind to a target is the IC$_{50}$ value, which is the concentration of the drug where the maximal biological function of the target is reduced to 50% under the given circumstances. The assays can be performed with or without competition of endogenous substrates, e.g. ATP in studies on TKIs. It can also describe the concentration of a drug which reduces the binding of a radiolabeled molecule to 50%\(^{(104)}\). IC$_{50}$ values are only applicable for drugs with an antagonistic action on the biological system.

**Lipophilicity** is important for the biodistribution of a drug. Molecules with a low lipophilicity are often excreted rapidly from the body and have a low to moderate tissue distribution. On the other hand, molecules with a high lipophilicity often have a high tissue distribution and good penetration of biological barriers but can also have a higher degree of non-specific binding due to their affinity for non-target protein structures and cell membranes. A moderate lipophilicity is often preferred for PET tracers, especially for brain imaging studies where sufficient transport across the BBB is essential\(^{(105)}\). The lipophilicity can be measured by liquid partition experiments in which the fraction of the substance that dissolves in a hydrophilic (water) phase is compared to that in a lipophilic (octanol) phase. However, lipophilicity is often estimated by computer simulations.
3.2.2 Transport Proteins and Tyrosine Kinase Inhibitors

Most of the clinically used TKIs appear to be either substrates and/or inhibitors of different transport proteins\(^{106}\), also referred to as efflux transporters. These transporters normally protect the cells by actively transporting potentially harmful substances from the cytosol across the cell membrane. The transporters have, however, also been shown to mediate multi-drug resistance in cancer, most likely acquired by selective pressure during treatment\(^{107}\).

The breast cancer resistance protein (BCRP/ABCG2) is a member of the adenosine triphosphate-binding cassette (ABC) transporter family and is a protein known to be able to rid the cells of a wide range of drugs and their metabolites. BCRP is mainly located in biological barriers such as the placental barrier and the BBB, but is also present in other tissues and has been isolated from breast cancer cell lines\(^{108}\). In mice, the expression of BCRP has additionally been confirmed in the adrenal and pituitary glands, ovary, testis, pancreas and adipose tissue\(^{109}\).

Another ABC transporter known to interact with TKIs is the P-gp or ABCB1. This transporter has been referred to as the gatekeeper of the BBB\(^{110}\) but is also, like BCRP, expressed on endothelial cell membranes in reproductive organs and can also be found in the papillary dermis of the skin\(^{111}\). High levels of P-gp expression in humans have also been reported in the adrenal glands, pituitary gland, colon, jejunum and heterogeneously distributed in liver and kidneys\(^{112}\).

It is interesting to compare the expression of these pumps to the uptake of the tracers evaluated in this thesis. Especially the high uptake of \((R)-[^{11}\text{C}]PAQ\) in the adrenals in rat (paper IV) could at least partially be explained by binding of the tracer to the possible P-gp expression here. It is also not unlikely that uptake in other structures can be due to binding to the efflux pumps e.g. the high uptake in the small structures in the head and neck of rat and mouse assumed to be the parathyroid glands in paper IV. Uptake in testis, liver, kidneys is also hard to explain with RTK expression only and an uptake contribution of ABC transporter protein binding could not be excluded. In a study on the interaction of the PAQ structure analog vandetanib, with P-gp and BCRP, it was suggested that the molecule is a substrate to the BCRP and solely an inhibitor of the P-gp\(^{113}\). However, it has been suggested that most of the TKIs are substrates to P-gp/BCRP at low concentrations while at higher concentrations they act as inhibitors\(^{106}\). This could possibly cause a discrepancy in the pharmacokinetics when a TKI is administered in micro dosing amounts (e.g. a labeled PET-tracer) as compared to the concentrations reached from therapeutic doses of the same substance or analogs.

As previously mentioned, TKI-based PET tracers have been used to study the interaction with the efflux proteins. When \([^{11}\text{C}]\)sorafenib was administered to genetically modified knock-out mice lacking the expression of both P-gp and BCRP, it resulted in a 3-fold increase of the tracer levels in brain tissue compared to WT-mice. This indicates that
sorafenib is a substrate to at least one of the efflux proteins\textsuperscript{(49)}. Similar results were obtained with \textsuperscript{11}C\textsuperscript{}gefitinib where a 8-fold increase in brain uptake was observed in the dual knock-out mice and radioactivity levels were also increased by the administration of Pgp and BCRP modulators in WT mice\textsuperscript{(114)}. Several other non-TKI-based PET tracers, both substrates and inhibitors, have been evaluated for the study of efflux transporter function or expression. These tracers are reviewed in\textsuperscript{(115)}.

**4 CANCER**

Cancer is the leading cause of death in the economically developed world\textsuperscript{(116)}. For example, approximately 23\% of all North Americans die from cancer. In the US, breast and prostate cancer are the most common malignancies found in women and men, respectively, while cancer to the lungs and respiratory system is the most common cancer related cause of death in both genders\textsuperscript{(117)}. Worldwide, breast cancer is the most frequently diagnosed cancer and the most common cause of cancer-related death in women (14\%, in 2008) while cancer to the lungs is the most common cancer death in men (23\%, 2008). An aging population and the adaptation of cancer-inducing behavior, especially tobacco smoking, in the economically developing countries will most likely further increase the incidence rates in the future\textsuperscript{(118)}.

Every cancer is unique. There are many different cancers and each type shares certain characteristics such as the tissue from which it originates and certain pathological hallmarks. Yet for every cancer the genetic background of the patient is different and so are, in most cases, the malfunctions and mutations preceding the cancer. However, in some cancers, strong hereditary contributions have been identified, including some types of breast\textsuperscript{(119)} and colon cancer\textsuperscript{(120)}. The hypothesis of tumor development suggests that cancer cells acquire their hallmarks of malignancy through an accumulation of gene activation and inactivation events over a long period of time. Important cancer hallmarks have been described in\textsuperscript{(121)} and include sustained proliferative signaling, resisting cell death (apoptosis), evasion of growth suppressors, replicative immortality, inducing angiogenesis, and activating invasion and metastasis (fig. 10). Finding a successful treatment often depends on characterization of those distinguishing features.

The specific hallmarks that are targeted by the tracers described in this thesis are the induction of angiogenesis (\textsuperscript{11}C\textsuperscript{}PAQ in papers II and IV) and the sustaining of proliferative signaling(\textsuperscript{11}C\textsuperscript{}PD153035 in papers I and III), both mediated by RTKs. The tracer molecules and target receptors are further described in sections 2.1 and 4.3, respectively.
Another typical cancer feature, which is investigated in relation to the $[^{11}C]$PAQ distribution in paper II and IV, is the deregulated cellular energetics, leading to a high glucose metabolism. This is considered one of the most important and common hallmarks of malignant transition. The reason behind the high glucose uptake is often referred to as the Warburg effect in which the cancer cells have switched to an aerobic glycolysis, using high amounts of glucose as a source of energy compared to normal differentiated cells, where oxidative phosphorylation is an essential part of the energy metabolism$^{(122)}$. The aerobic glycolysis in the proliferating cells governs the growth of biomass and produces by-products such as lactic acid, leading to a low pH environment in the inner parts of the tumor$^{(123)}$. Cancer diagnosis based on glucose utilization is the main clinical application of PET today and is described in more detail in section 4.4.

4.1 Metastasis

Cancer that has spread from the primary tumor site to another non-adjacent part of the body is metastatic. The new occurrences of disease are referred to as metastases. Metastatic disease substantially worsens patient prognosis and is the single most important factor in cancer-related deaths$^{(124)}$.

For a cancer to become metastatic the cells have to acquire several characteristics, including the ability to survive outside of the primary tumor and to be able to colonize new organs with sustained capabilities of growth and angiogenesis. Furthermore, the cells can acquire
additional deadly traits in the new tissue and this biological heterogeneity of cancer cells in the primary tumor and in metastases makes the disease harder to treat and more unpredictable\textsuperscript{(125)}. It is known that cancers in certain organs have preferences in their pattern of distant spread, e.g. prostate cancer often metastasizes to bone tissue while initial metastasis from colorectal cancer is mainly in the liver\textsuperscript{(124)}. In paper IV we show indications that \((R)\)-[\textsuperscript{11}C]PAQ was able to visualize lung metastases based on VEGFR2 expression in and around the growing lesion.

4.2 Angiogenesis

Angiogenesis is the formation of new capillaries by cellular outgrowth from existing microvessels. The process is essential for a cancer tumor to grow beyond the size of 1-2mm in diameter\textsuperscript{(126)}. The balance of vascularization is regulated by pro- and anti-angiogenic factors. In cancer, the angiogenic switch can be turned on by factors such as low oxygen levels, low pH or hypoglycemia. The rate of angiogenesis is determinative for the rate of tumor growth and has been associated with metastatic spread\textsuperscript{(127)}. In papers II and IV, the aim was to evaluate the ability of \((R,S)\)-[\textsuperscript{11}C]PAQ and \((R)\)-[\textsuperscript{11}C]PAQ, respectively, to visualize areas with active angiogenesis. This is based on the fact that the expression of the target receptor, VEGFR2 (described in section 4.3.2), is highly associated with angiogenic processes\textsuperscript{(128)}.

4.3 Receptor Tyrosine Kinases

At least 58 different unique genes encoding for RTKs have been identified in the human genome\textsuperscript{(129)}. RTKs are cell surface proteins that consist of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular domain with TK activity. The main function of the TK is to transfer phosphate via the tyrosine kinase domain to intracellular proteins\textsuperscript{(130)}. Receptor signaling is generally initiated by ligand binding to the extracellular domain of the receptor. Subsequent receptor dimerization and phosphorylation of the intracellular TK domain will trigger a signaling cascade of biochemical messages leading to diverse cellular responses (fig. 11). The signaling is usually terminated by internalization of the receptor complex\textsuperscript{(131)}.

The receptor function is usually well controlled in normal cells but increased RTK expression or mutated RTKs can become potent oncoproteins since they are key mediators of fundamental diverse cellular processes such as proliferation, migration, metabolism, differentiation and cell survival. Numerous cancers display an abnormal RTK function or expression and RTKs are therefore very important therapeutic targets\textsuperscript{(131, 132)}. 
Figure 11. General signal activation of RTKs (I) with extracellular ligand binding, receptor dimerization and intracellular phosphorylation of the TK-domains (II).

4.3.1 Epidermal Growth Factor Receptor

EGFR (erbB1/HER1) is a RTK that is normally expressed in epithelial, mesenchymal and neuronal tissue. The receptor is well preserved across species and plays a major role in tissue maintenance and repair, development and cell differentiation\(^{(133, 134)}\).

Dysregulation of EGFR expression or function is associated with several key features of cancer including initial oncogenic transformation, increased aggressiveness, invasiveness and therapy resistance\(^{(135, 136)}\). Up-regulation of EGFR expression and/or function is a hallmark of many human malignancies including some of the most lethal cancers such as breast cancer, gliomas and lung cancer\(^{(137)}\). Proportions of tumors expressing EGFR in different cancer types are given in table 2.

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Proportion of tumors expressing EGFR (%)</th>
<th>Cancer</th>
<th>Proportion of tumors expressing EGFR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glioblastoma</td>
<td>40–50</td>
<td>Renal Cell</td>
<td>50–90</td>
</tr>
<tr>
<td>Head and neck</td>
<td>80–100</td>
<td>Prostate</td>
<td>40–80</td>
</tr>
<tr>
<td>Lung</td>
<td>40–80</td>
<td>Bladder</td>
<td>53–72</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>30–50</td>
<td>Cervical</td>
<td>54–74</td>
</tr>
<tr>
<td>Colorectal</td>
<td>25–77</td>
<td>Ovarian</td>
<td>35–70</td>
</tr>
<tr>
<td>Esophageal</td>
<td>71–88</td>
<td>Breast</td>
<td>14–91</td>
</tr>
</tbody>
</table>

Table 2. Proportion of common solid tumors expressing EGFR. Numbers were reported in several different studies which are reviewed in\(^{(135, 138)}\).
Mutations of the TK domain of the EGFR leading to increased susceptibility to targeted treatment with TKIs have been found in many lung cancer patients and are considered an important prognostic factor in NSCLC\(^{(139)}\). When cancer with the activating mutations (exon 19 deletion or exon 21 L858R point mutation) is treated with erlotinib, the overall survival of patients is significantly increased, compared to treatment with traditional cytostatics\(^{(140, 141)}\). This implies that screening for mutational status in lung cancer patients before treatment is initiated should be important. Mutation frequency has been found to depend on gender, ethnic origin and type of NSCLC. The differences are rather striking with the highest proportion of mutations found in female Japanese women with lung adenocarcinomas (54%) while only a few percent of the male patients from the United States carry an activating mutation\(^{(139)}\). The same mutations have also been found in patients with squamous cell carcinomas of the head and neck and the mutation frequency (7.3%) roughly correlates with the proportion of responders to targeted TKI therapy\(^{(142)}\). In tumors from patients with colorectal cancer or glioblastoma, the mutational frequency was found to be very low and it was concluded that these patient groups would not likely respond well to single TKI therapy\(^{(143)}\).

Even though some patients have benefited greatly from the TKI treatment, most of them develop an acquired resistance to the therapy leading to progress or recurrence of disease. Two different mutations have so far been found to harbor this resistance. One is a mutation in exon 20 (T790M) of the TK domain and the other is an amplification of the MET proto-oncogene. Both mutations have been shown to be absent in the tumor samples taken before treatment with EGFR targeted TKI therapy\(^{(144, 145)}\) and are therefore presumed to be generated by the selection pressure induced by the TKI treatment.

Together with the above described mutations, a high EGFR gene copy number resulting in an overexpression of EGFR, is generally considered a positive predictive factor for EGFR targeted therapy\(^{(146)}\)\(^{146}\). The opposite has been found for the KRAS mutation which is considered an important negative factor, predicting a lower response rate to EGFR-targeted therapy\(^{(147, 148)}\).

### 4.3.2 Vascular Endothelial Growth Factor Receptor 2

VEGFR2 (KDR/Flk-1) is a RTK which, together with its ligands, VEGF A, C and D, mediates processes involved in the vascularization of tissue\(^{(7, 128, 149)}\). VEGFR2 is normally involved in processes related to wound repair, embryogenesis and in maintaining normal tissue functions\(^{(128)}\). Its involvement in cancer development has been studied and well described. Initially the receptor is expressed during the angiogenic switch, a pivotal process required for growth beyond the size of microtumors. During further growth of tumors, expression of VEGFR2 is essential for creating the supporting microvascular network in and around the growing tumor\(^{(150, 151)}\). The VEGFR2 is often expressed on the endothelial cells associated with the vasculature in the tumors and surrounding tissue and not as commonly on the tumor cells\(^{(152)}\).
In several different cancer types, including breast, prostate, colorectal and cancers to the head and neck, a high expression of VEGFR2 has been found in tumor tissue. In many cases a higher VEGFR2 expression is considered a negative prognostic factor for the patient and a sign of a cancer with invasive behavior and higher metastatic potential\textsuperscript{(153-158)}.

4.4 Diagnosis with Positron Emission Tomography

PET is used in clinical oncology to locate and characterize cancer based on specific biochemical processes and biomarker expressions that are crucial for the development, growth and spread of the cancer. \textsuperscript{[18]F}FDG is the most commonly used tracer in diagnostic oncology\textsuperscript{(159)}. \textsuperscript{[18]F}FDG is a D-glucose analog that is taken up and accumulated in tissue with high glucose demands\textsuperscript{(160)}. In a healthy individual, high uptake is usually observed in organs with a high glucose utilization such as the brain and heart and also in the urinary bladder due to the renal excretion. As previously described, many cancers have an abnormally high uptake of glucose and can therefore be located and the metabolic status estimated using \textsuperscript{[18]F}FDG. Elevated levels of \textsuperscript{[18]F}FDG uptake have been correlated to cancers that grow more aggressively and to poorer patient prognosis\textsuperscript{(161-164)}. The tracer is also used in monitoring response to treatment\textsuperscript{(165, 166)}, staging and for detection of primary tumors and distant metastasis\textsuperscript{(167)}. In papers II and IV \textsuperscript{[18]F}FDG was used in combination with \textsuperscript{[11]C]PAQ for tumor localization and also as a comparison of tumor visualization and characterization abilities.

Other clinically used tracers in diagnostic oncology are 3′-deoxy-3′-\textsuperscript{[18]F}fluorothymidine (\textsuperscript{[18]F}FLT), \textsuperscript{[18]F]misonidazole (\textsuperscript{[18]F}MISO), \textsuperscript{[11]C]acetate, \textsuperscript{[11]C]methionine and \textsuperscript{[18]F]fluoride. \textsuperscript{[18]F}FLT is used to assess proliferation in suspected malignancies or recurrent cancers\textsuperscript{(168, 169)}. \textsuperscript{[18]F]MISO is trapped in cells with low oxygenation and can thus be used to image hypoxic areas of tumors and surrounding tissue. Tumors grown in hypoxic conditions are often more resilient to therapy and can be more aggressive\textsuperscript{(170)}. \textsuperscript{[11]C]Methionine and \textsuperscript{[11]C]acetate are endogenous molecules used to detect abnormal metabolism in areas where \textsuperscript{[18]F]FDG has a high normal physiological uptake, e.g. brain and prostate (close to the urinary bladder) respectively\textsuperscript{(171, 172)}. \textsuperscript{[18]F]Fluoride is incorporated in skeletal tissue, especially in newly formed tissue, and can therefore be used to identify skeletal metastasis\textsuperscript{(173)}. 

25
4.5 Tyrosine Kinase Inhibitors in Treatment

The group of drugs called TKIs act as competitive inhibitors of the ATP-binding sites at the catalytic intracellular TK domain of RTKs, thereby preventing cellular signaling by the receptors (fig. 12). Although the molecules all share the same mechanism of action they differ in the spectrum of targeted RTKs and also in the chemical structure. The two tracer candidates evaluated in this thesis, \( [^{11}\text{C}]PD153035 \) and \( [^{11}\text{C}]\text{PAQ} \), share the 4-AQ structure with some of the clinically used substances and thus likely some of the pharmacokinetic characteristics such as being metabolized by CYP3A4 (table 3)\(^{(88, 174)}\).

Figure 12. TKI-blocking of intracellular RTK-signaling.

The TKIs can be further divided into a few subgroups. The first generation TKIs, including erlotinib and gefitinib, have a specific binding to only one RTK or a small group of RTKs\(^{(64)}\) while other, later developed TKIs have multi-targeting properties such as cabozantinib\(^{(175)}\) and axitinib\(^{(176)}\).

Second generation TKIs (dacomitinib, deratinib, lapatinib and afatinib) that bind irreversibly to HER2- and/or EGFR-TKs, have been indicated for treatment of patients with acquired resistance for erlotinib and gefitinib - especially for resistance mediated by the T790M mutation. Progress in these studies has been reviewed in\(^{(177, 178)}\).
Table 3. Overview of some TKIs approved for clinical and veterinary use as monotherapies or in late stage clinical trials in the US and/or Europe. The main route of metabolism reported in the literature as well as the target RTK(s) and approved indications are also listed. Text in italics describes indications currently evaluated in clinical trials.
4.6 Targeted Treatment and Personalized Therapy

When the TKI imatinib was approved for treatment of chronic myeloid leukemia (CML) in 2002, it was the first clinically used drug that targeted a cancer-specific protein. The oncogene BCR-ABL is created by a genetic mutation and expresses the Bcr-Abl protein, which has a strong TK activity and is highly involved in the genesis of CML\(^{(202)}\). Since this pharmacological breakthrough, several drugs with targeted therapeutic properties have been developed, including many of the TKIs previously described. Targeted cancer therapies provide an opportunity to individualize cancer treatment, especially when a target is present in some but not all tumors of a certain type, as is the case for exon 19 mutated EGFR in NSCLC\(^{(139)}\). Being able to accurately identify tumor characteristics such as sensitizing mutations or amplified expressions can greatly increase the success of targeted treatment, which eventually may be tailored to counter the unique targets expressed by the patient’s cancer. Targeted cancer therapies are, in addition, often more selective for cancer cells than normal cells, thus reducing side effects and improving quality of life. Furthermore, applications predicting response to therapy can have both economical and health benefits since patients that are not likely to respond can instead be subjected to other treatment strategies without losing valuable time to an ineffective treatment and a progressing disease.

However, diagnostic strategies based on several methods are, in most cases, required to pinpoint the weaknesses of the disease. PET with \(^{18}\text{F}\)FDG or \(^{18}\text{F}\)FLT and possibly more target specific tracers, such as TKIs or monoclonal antibodies, in combination with other imaging modalities, genetic and pathological analysis of available biopsies and measurements of blood markers can more accurately characterize the lesions and estimate the spread and status of disease. Even though there is a great knowledge on how a personalized medicine can be achieved, the implantation of scientific discoveries into routine healthcare care is often slow, and new techniques can be costly to implement\(^{(203)}\).

Determining whether molecular dysfunctions and therapeutic responses can be identified, predicted and quantified in pre-clinical models with radiotracers such as \(^{11}\text{C}\)PAQ is a key step toward a possible future implementation in human studies. These new tracers will not solve all diagnostic problems and will most likely never be as important as a general imaging biomarker like \(^{18}\text{F}\)FDG in diagnostic oncology, but can nevertheless offer new valuable tools for the biological characterization of patients if used wisely. Among the TKI-based PET-tracers, \(^{11}\text{C}\)erlotinib is the most convincing so far, due to the ability to predict treatment response based on mutational status of the cancer rather than levels of EGFR expression\(^{(36, 37)}\).

The greatest limitation of the targeted therapies is, for many treatments, the inevitable development of drug resistance\(^{(144)}\). It is, however, possible that tracers successful in finding sensitizing hallmarks can also have some use in excluding patients from targeted therapy, e.g. if the patient is examined after some time on the targeted therapy, when a resistance is more likely acquired. So far a number of promising PET tracers are being evaluated, not
only those based on TKIs, but also antibodies and small peptides, as discussed under section 2.2. Hopefully this research will produce additional tools that can be used in improving cancer care.

5 CANCER MODELS

5.1 Implants and Injection of Tumor Cells

In papers II and III, SC tumors were used as disease models for the evaluation of the tracers. These tumors grow under the skin after the injection of tumor cells. Usually a suspension of several million cells is injected, of which a small fraction will begin to grow and establish a tumor. The tumor take and rate of growth are dependent on the amounts and characteristics of the tumor cells, host animal genetics and the location of the injection site. Although not a very realistic model of human tumor growth, SC tumors offer a platform easy to control for initial studies in tumor tracer development. In paper II we additionally used a intraperitoneal (IP) model of tumor development where the cells were injected into the peritoneal cavity and subsequent tumor development followed by $^{18}$F]FDG PET.

The TUBO cell line used in paper II was originally isolated from a spontaneous mammary tumor in a BALB NeuT transgenic mouse. The tumors grow relatively fast and express high levels of HER2 RTKs on the cell surface$^{(204)}$. The B16F10 cell line is a widely used mouse melanoma model characterized by aggressive growth and metastatic spread$^{(205)}$. The B16F10 AST cells express the local angiogenesis inhibitor angiostatin and has been used in studies on vasculature and metastasis development$^{(206)}$. Primary tumors of the latter often tend to develop a necrotic core.

The A431 cell line used in paper III originates from a human fibrosarcoma tumor and expresses extremely high levels of EGFR$^{(205)}$ due to gene amplification$^{(207)}$. Because of the human origin the cells need to be injected into immunocompromised animals in order to avoid rejection of the alien cells - xenografts.

5.2 Genetically Modified Animals

Cancer models in which the animals are genetically modified and spontaneously develop tumors offer a unique opportunity to study the disease progression from early malignant transition to late stage disease with possible metastatic spread. The models provide an interesting evaluation platform for novel PET tracers such as $^{11}$C]PAQ in preclinical imaging.

In paper IV we used the MMTV-PyMT transgenic mouse model of metastatic breast cancer. In this model the expression of the polyoma middle T (PyMT) oncoprotein is controlled by the mouse mammary tumor virus (MMTV). The model has been well characterized and proven to have a good translational potential with features such as gradual progression from hyperplasia to later stages with significant stromal infiltration and pulmonary metastasis.
In the mice with the FVB/NJ genetic background, progress latency times are shorter compared to other genetic backgrounds. Mammary tumors and subsequent metastatic disease to the lungs develop over a time course of 12-15 weeks with hyperplasia normally found in the mammary glands around weeks 4-5\textsuperscript{(208)}. The PyMT protein activates the same signaling pathways as the HER2 RTK which is found in over 30\% of breast cancer malignancies\textsuperscript{(209)}. Additional features observed in the model include an angiogenesis mediated transformation of micrometastasis to macrometastasis, which is suggested to be regulated by endothelial progenitor cell infiltration\textsuperscript{(210)}.

5.3 Animals and Ethical Considerations

In the PET experiments performed in the studies of this thesis, no animals died from complications during the experimental procedures. At all times, animal health and well-being were closely monitored, both before, during and after the experiments. The guidelines for animal handling and monitoring at the Karolinska Institutet were strictly applied.

PET is a great example of a technique that can facilitate implementation of the three R’s; reduction of the number of animals, replacement of experimental procedures and a refinement of research in order to lessen pain and suffering\textsuperscript{(211)}. PET experiments generate tremendous amounts of information and the non-invasive nature of the technique allows for serial imaging of individual animals. If used in well-designed experimental setups with good planning, this technique can greatly reduce the number of animals needed to be able to draw scientific conclusions on a statistical basis. PET is a highly quantitative technique that can replace other experiments, e.g. in studies of biodistribution and treatment response and, at the same time, decrease both the number of animals needed and the potential pain and suffering the animals experience.
AIMS OF THE THESIS

The overall aim of this thesis was to evaluate the pharmacokinetic properties and RTK imaging abilities of two TKI based PET tracers.

The specific aims of each paper were as follows:

Paper I: To investigate the *in vitro* metabolism of [6-\(^{11}\)C-methyl]PD153035 and [7-\(^{11}\)C-methyl]PD153035 and to identify possible radioactive metabolites.

Paper II: To evaluate the pharmacokinetics of (R,S)-[^\(11\)C]PAQ and the VEGFR2 imaging properties in tumor xenograft models in mice.

Paper III: To assess the *in vivo* metabolism of [6-\(^{11}\)C-methyl]PD153035 in rats and to investigate whether drug induced alterations of metabolism affects the biodistribution in healthy animals and radioactivity uptake in EGFR overexpressing tumors.

Paper IV: To evaluate the pharmacokinetics of (R)-[^\(11\)C]PAQ and to assess imaging abilities in the MMTV-PyMT breast cancer model.
RESULTS AND COMMENTS

*In vitro* Metabolism of $[^{11}\text{C}]$PD153035 (Paper I)

In paper I *in vitro* techniques were used to examine the hepatic metabolism of $[^{11}\text{C}]$PD153035 (section 2.1.1). Previous metabolism studies on PD153035 used at therapeutic doses in mice\(^{212}\) revealed that four metabolites were present in plasma and in A431 tumor tissue after a single IP dose of PD153035. Two of these metabolites were identified as the 6- and 7-O-desmethyl compounds, the other two metabolites were not identified. These observations raised questions about the possible implications of the labeling position on the pharmacokinetics of the tracer, which we have attempted to answer with the experiments performed in paper I.

PD153035 labeled with $^{11}$C in either position 6 or 7, was incubated with either human or rat liver microsomes over 60 min. The microsomes were activated by keeping a temperature of 37° C and adding NADPH prior to the addition of $[^{11}\text{C}]$PD153035. Aliquots were removed from the solution during the incubation, filtered and analyzed with radio-HPLC. Fractions were collected during the analysis and measured in a gamma counter for increased sensitivity.

The main metabolite found in all assays was an N-oxide, while metabolites formed by O-demethylation of the methoxy group in position 7 were only observed after incubation with human liver microsomes (fig. 13).

![Chemical Structures](image)

**Figure 13.** Radiometabolites observed after liver microsome incubations of [6-0-$^\text{11}$C-methyl]PD153035 and [7-0-$^\text{11}$C-methyl]PD153035.

The N-oxide was identified by LC-mass spectrometry after scaling up the assays and pooling the HPLC-fractions containing this unidentified metabolite. The 7-O-desmethyl metabolite was identified by its retention time on HPLC that correlated to non-labeled reference. Polar radiometabolites generated during incubation with [7-0-$^\text{11}$C-
methyl]PD153035 were assumed to be products from the same demethylation reaction that produced the radioactive 7-O-desmethyl metabolite from [6-O-11C-methyl]PD153035 (fig. 13).

Interestingly, no labeled products arising from 6-O demethylation were detected in this in vitro assay. This can perhaps be explained by the lack of certain enzymes or essential cofactors in the microsome preparations since under in vivo conditions these metabolites were produced both in mice^{212} and as we found later, in paper III, probably also in rats.

Based on the extensive literature on TKI metabolism (see section 3.1.2), we assumed that CYP-enzymes present in the isolated microsomes were most likely responsible for the metabolite formation. The function of these enzymes depends on oxygen and NADPH as previously described in section 3.1. A lack of one of these might explain the decrease in metabolite formation rate from around 20-30 min (fig. 14). We did not explore whether subsequent additions of NADPH would have extended the metabolism, but we presume it is very likely. The relatively low SR obtained with the “wet” method used for radiolabeling in these experiments can also have contributed to the depletion of energy in the incubation solution since more PD153035 was being processed. The intermittent swirling of the solution should have maintained adequate oxygenation levels.

We concluded that, if these in vitro results were predictive of the [11C]PD153035 metabolism occurring in vivo, estimations of the EGFR receptor density based on
radioactive uptake could be problematic and the choice of labeling position would be of decisive importance. Since the methoxy group at position 7 was found to be more prone to demethylation, our conclusion was that labeling in this position may possibly be more favorable when a faster clearance of radiometabolites is desired, since relatively more of the polar radioactive metabolites are formed rather than the more lipophilic 6-O metabolite. Furthermore, both desmethyl metabolites have a rather high affinity for the EGFR-TK ($K_i = 25-168$ pM), though they have been shown to clear from tumor tissue faster than the parent compound$^{212}$.

**In vitro and In vivo Evaluation of (R,S)-[11C]PAQ (Paper II)**

By the time of this study we had acquired the microPET Focus 120® PET-camera allowing us to do preclinical oncology studies in mice. In this paper we reported the first radiosynthesis of (R,S)-[11C]PAQ (section 2.1.2). The synthesis of the precursor molecule was performed by previously published methods as described in paper IV. We realized that, by using the racemate, some pharmacokinetic questions might be difficult to answer due to possible differences in the behavior of the two stereoisomers. However, if positive imaging results were obtained, we intended to follow up these initial studies by using only the R-isomer, which was accordingly done later in paper IV.

Performing the in vitro metabolism assays, generally by the same methods as described in paper I, we observed a very low metabolism which also was assumed to be correlated with the negligible excretion noticed in the in vivo biodistribution studies. The radioactivity distribution observed in the PET studies showed high uptake in lung, kidneys and liver, which is consistent with the VEGFR2 expression in these organs$^{213}$. In the kidneys we were able to show with PI that the radioactivity uptake correlated well with the expression pattern of VEGFR2. The distribution and excretion pattern in mice was thus comparable with that of the structural analog vandetanib, which is a metabolically stable drug with low excretion and a very long half-life of ~30 h$^{214}$ and ~120 h$^{93}$ in mouse and man, respectively.

The blocking experiments showed a rather robust effect with a considerable decrease in lung, liver and kidney uptake after pretreatment with (R,S)-PAQ. This indicates that the unlabeled drug was able to hinder the binding of (R,S)-[11C]PAQ to targets in this tissue, presumably to VEGFR2. The results also imply that SR levels can be important when the tracer is used in quantification purposes - depending on VEGFR2 levels in the tissue of interest. Displacement effects using the same amounts (5 nM and 50 nM internal concentration) of (R,S)-PAQ were not measurable when the drug was administered 15 min into the PET scan. An irreversible or low $K_i$ binding to the VEGFR2 and/or an irreversible binding to other targets could explain this lack of displacement. In the initial studies$^{61}$ it was not reported whether (R,S)-PAQ binds reversibly or irreversibly to the VEGFR2. However, due to the lack of structural elements, such as acrylamides, that are normally used to achieve irreversible covalent binding by TKIs$^{215}$, it can be reasonable that (R,S)-PAQ would bind reversibly to VEGFR2.
Of the cell lines (TUBO, B16F10 and B16F10 AST) used here for the imaging purposes, B16F10 and B16F10-AST had both previously been characterized in angiogenesis studies\(^{(206, 216)}\). They were not chosen as positive controls based on high VEGFR2 expression, but rather as a model of tumor angiogenesis.

Uptake of \((R,S)\)-\([^{11}\text{C}]\)PAQ in the SC tumors was relatively low and with a heterogeneous distribution of radioactivity. The tumors that grew after TUBO cell injection together with the matrigel matrix were not discernible with \((R,S)\)-\([^{11}\text{C}]\)PAQ while the highest uptake was observed at the rim of tumors grown with the B16F10 cell line. The B16F10-AST cells generated tumors with a necrotic core, displaying very low uptake at the center of the tumors while uptake was higher at the viable outer border, which was visualized with both \((R,S)\)-\([^{11}\text{C}]\)PAQ and \([^{18}\text{F}]\)FDG PET. While we did not have the opportunity to perform anatomical imaging with CT or MRI at this time, \([^{18}\text{F}]\)FDG served well as a marker of tumor locations and volumetric estimations since the uptake was generally homogenous in the tumors. \([^{18}\text{F}]\)FDG accumulated even in the matrigel plugs that lacked uptake of \((R,S)\)-\([^{11}\text{C}]\)PAQ.

The SC tumors were characterized by a rather isolated, non-invasive growth with a few large blood vessels connected to the host (fig. 15 I). IHC also confirmed that VEGFR2 was present in appreciable levels only at the rim of the tumors. These observations led us to try another strategy in which tumor cells were injected IP. We could now observe a more invasive growth with a richer vasculature in tumors that grew mainly out from the pancreas and surrounding adipose tissue (fig. 15 II). These lesions displayed a more pronounced uptake of \((R,S)\)-\([^{11}\text{C}]\)PAQ with intense hotspots observed in PET and PI, which also correlated to the distribution of VEGFR2 as assessed with IHC.

![Figure 15](image.png)

Figure 15. To the left (I), a SC tumor 15 days after injection of matrigel-embedded TUBO cells. To the right (II), an IP tumor, 11 days after injection of B16F10 melanoma cells. The areas surrounding the tumors have been blurred in the photos.

In summary, the imaging results with \((R,S)\)-\([^{11}\text{C}]\)PAQ were promising, especially in the IP model. We were also encouraged by the good correlation between \textit{ex vivo} VEGFR2 expression and radioactivity levels in tumor tissue, assessed with IHC and PI, respectively. Moreover, the \textit{in vitro} metabolism study and PET biodistribution studies showed that we
were dealing with a molecule with good metabolic stability and distribution patterns allowing for tumor imaging of most parts of the body, with possible limitations to the liver and kidneys due to high normal uptake levels in these organs.

**In vivo Metabolism of [6-O-11C-methyl]PD153035 in Rat (Paper III)**

In paper III the *in vivo* biodistribution and metabolism of [6-O-11C-methyl]PD153035 was examined in rat. In addition we investigated whether biodistribution and tumor imaging properties were affected by alterations in metabolism. Based on the *in vitro* metabolism results with rat liver microsomes in paper I, we expected to see formation of the N-oxide metabolite, although the extent of metabolism was difficult to predict. Recent studies in humans\(^{(57,58)}\) increased the importance of performing these studies since we had concluded (paper I) that the *in vivo* metabolism could have possible implications for tumor imaging based on EGFR expression.

We chose to use the tracer labeled in position 6 for these experiments. This was based on the results from the *in vitro* studies in paper I where we found that the tracer may be prone to demethylation in position 7 only. On the reversed phase radio-HPLC-system used in these assays it should then be more reliable to estimate the extent of 7-demethylation by the levels of the corresponding identified radiometabolite instead of trying to analyze the very polar radiometabolites generated from [7-O-11C-methyl]PD153035. These would elute very early and therefore possibly co-elute with other hydrophilic radiometabolites. The HPLC-setup was a compromise between retention times and separation between the metabolites (fig. 16). Intact [6-O-11C-methyl]PD153035 eluted at around 11-12 min which proved to give us just enough time to prepare and analyze the two separate blood samples (10 and 30 min) and the urine sample (60 min) for each animal without the radioactivity levels becoming too low for subsequent gamma counter measurements.

![Figure 16](image-url) [11C]PD153035 and radioactive metabolites. A typical radiochromatogram (smoothed) reconstructed after measurements of radioactivity of HPLC-fractions.
Results obtained in healthy rats showed an extensive in vivo metabolism with relative plasma concentrations of only ~10% intact [6-O-\(^{11}\)C-methyl]PD153035 at 10 min after IV injection. We were somewhat surprised to see that not only the N-oxide was present in blood samples, but also the 7-O-demethylated radiometabolite together with polar metabolites possibly generated by demethylation of the \(^{11}\)C-labeled methoxy-group in position 6. The metabolites were excreted by both renal and hepatobiliary pathways with the more polar metabolites being excreted predominantly to the urinary bladder. Except for very low levels of \([^{11}\text{C}]PD153035\) found in feces, the excreted radioactivity consisted only of metabolites.

We also demonstrated that the metabolism could be altered with drug-induced enzyme inhibition. Selective inhibition of hepatic cytochrome P 450 enzymes in the subfamilies CYP2D and CYP3A, with quinidine and ketoconazole (see section 3.1), respectively, altered the generated amounts of all three metabolites. The relative levels of intact [6-O-\(^{11}\)C-methyl]PD153035 were increased after both treatments. The largest difference was observed after CYP3A inhibition with a 3-fold increase of parent tracer compared to controls. Only small differences in biodistribution between the treated and control animals were observed in the PET data of blood, kidneys, bladder and intestines. However there was a tendency to changes in radioactivity concentrations in the liver – an organ responsible for metabolism of the tracer and also known to express significant amounts of EGFR even in adult animals\(^{(217)}\). The control animals reached a higher maximum liver concentration of radioactivity and also exhibited a faster elimination from the liver.

The uptake in the EGFR overexpressing A431 tumors after IV injection was low, reaching levels that were comparable to reference muscle tissue and blood. The radioactivity levels in blood, measured with a ROI drawn over the vena cava inferior, was similar in animals treated with both enzyme inhibitors and the non-treated animals. Thus it can be assumed that the amounts of intact tracer that possibly could reach the tumor via the blood was higher in the treated animals and it was therefore a surprise that tumor uptake levels were actually lower in these animals. This suggests that the hydrophilic radiometabolites contribute to the total uptake of radioactivity in the tumor; an uptake most probably influenced by various parameters such as perfusion, EGFR expression and individual metabolic capacity and blood clearance.

The local IA injection of [6-O-\(^{11}\)C-methyl]PD153035 was performed as a proof of principle and though only one experiment was performed the results were interesting and informative. As expected, the levels of radioactivity reaching the tumor after the IA injection were considerably higher compared to the IV injections. It was, however, interesting that the tumor to muscle ratio was increased after IA administration. The ratio was nearly doubled compared to that after IV injections, 30 min after administration, when the time activity curves had leveled out. This indicates that a tumor specific EGFR binding is present, if the intact tracer has a chance to reach the tissue.
Previous studies on distribution to A431 tumors in mice showed that PD153035 was present in tumor tissue up to 12 h after a bolus dose\(^{(212)}\). As opposed to our poor tumor imaging results with tracer amounts of \([6-O^{11}\text{C}-\text{methyl}]\text{PD153035}\), this may be explained by the larger amounts of PD153035 saturating metabolic enzymes and other binding sites, e.g. in liver, which results in a higher concentration and subsequent retention of intact drug in tumor tissue. It is possible that a pretreatment with unlabeled PD153035 would have increased EGFR-specific uptake levels in tumor tissue after IV injection of the tracer. This strategy has been successfully used with EGFR targeting Affibodies, as discussed in section 2.2. Analogously, administration of \([^{11}\text{C}]\text{PD153035}\) with a considerably lower SR would, hypothetically, improve tumor imaging properties. This can explain the reported correlation between EGFR expression and uptake of \([^{11}\text{C}]\text{PD153035}\) in tumor xenografts in mice\(^{(56)}\). The tumor to muscle ratios in that study were highest at 30 min post injection with values of approximately 4 in the xenografts expressing high levels of EGFR. In our study we observed a tumor to muscle ratio of 2.2 and 1.2 for the A431 tumors, 30 min after the IA and IV injections, respectively. It is however worth to notice that the reported uptake levels were low in the mice tumor xenografts, with a maximum uptake of 0.14%ID/g, which is comparable to our results in the A431 xenografts after IV injection. The reported SR in\(^{(56)}\) was in the range of 23 to 45 GBq/μmol at end of synthesis, and consequently less at injection. The same range in SR for \([^{11}\text{C}]\text{PD153035}\) was presumably used in the dosimetry and tumor imaging studies in humans\(^{(57, 58)}\) since the same synthesis methods were used, although neither the SR values nor the labeling position were reported in these papers. Our SR in paper III of 500 GBq/μmol at injection was thus, at least, around 20 times higher. This difference in SR may contribute to the differences observed in tumor uptake in these studies. However, this does not change the fact that the extensive metabolism is most likely a crucial and severely limiting factor for EGFR quantification with \([^{11}\text{C}]\text{PD153035}\).

**(R)**-[\(^{11}\text{C}\)]PAQ in the MMTV-PyMT Model (Paper IV)

In this study the pharmacokinetics of \((R)-[^{11}\text{C}]\text{PAQ}\) was studied in rat and mouse, although the primary aim was to evaluate ability of the tracer to visualize angiogenesis associated with tumor growth. Since \((R)-[^{11}\text{C}]\text{PAQ}\) had a 10-fold lower IC\(_{50}\) and a 200-fold higher affinity to the VEGFR2 versus the EGFR compared to the S-isomer, we were confident that the studies would be conducted with a better imaging candidate than in the studies in paper II in which the racemate was used. Starting from the \((R)-\text{tert-butyl 3-(hydroxymethyl)piperidine-1-carboxylate, the (R)-desmethyl precursor of (R)-[^{11}\text{C}]\text{PAQ}\) was synthesized in 3 steps as described in the supplementary material of paper IV.

The radio-HPLC based metabolite assay, described in paper III, was used to investigate the metabolism of the tracer in rats and it was found that the tracer was fairly metabolically stable for 60 min after injection. At 30 min and 60 min after injection, 77% and 57%, respectively, of the radioactivity in plasma was still intact \((R)-[^{11}\text{C}]\text{PAQ}\). Biodistribution of the \((R)-\text{isomer in mouse was similar to that of the racemate reported in paper II. The largest difference was observed in the kidney which displayed a higher uptake of the racemate.}
The distribution in rat followed the same pattern as in mouse with high levels of radioactivity in lung, kidney and liver. We could also observe high levels of uptake in the adrenal glands and in several smaller structures in the head and neck region, some that were identified as the parathyroid glands. In the liver, adrenals and parathyroid glands the radioactivity was retained or accumulated during the 60 min scan while in the lung and kidney the levels decreased after the initial peak.

We used the previously described MMTV-PyMT model of metastatic cancer (section 5.2) in which several stages of cancer are present at the same time. This allows for imaging of tumors in early development and metastatic disease in the same animal. We performed (R)-\[^{11}\text{C}\]PAQ PET in several animals, at the age of 11-15 weeks when they had confirmed mammary gland tumors and they were likely to have developed pulmonary metastasis. Since the development of disease in this model exhibits individual variations, tumor size and spread were essentially independent of age in the animals used in this study.

Mammary glands with primary tumors were clearly visible in PET, exhibiting a slow but steady accumulation of radioactivity over the 60 min PET scans. From 10 to 60 min SUV values were generally doubled in the tumorous mammary gland tissue while no increase was noticeable in the mammary gland region of the WT animals. PI combined with IF confirmed that uptake of radioactivity correlated well with VEGFR2 expression in the primary tumors. An even better correlation was obtained when expression of CD31 was mapped over VEGFR2 and radioactivity distribution. CD31, also known as platelet endothelial cell adhesion molecule, is expressed at vascular endothelial cell-cell borders and thus its expression indicates the presence of blood vessels. In most areas a co-expression of VEGFR2 and CD31 was observed down to the levels of individual blood vessels. However, in some areas of the tumor, with high expression of VEGFR2 but low in CD31, the radioactivity uptake was relatively low. This may be explained by a locally low perfusion caused by the lack of functioning blood vessels, as indicated by the low levels of CD31 expression.

The lungs from the animals displaying distinct focal lung uptake of (R)-\[^{11}\text{C}\]PAQ were sectioned for IF analysis. By sectioning the whole lung we were sure not to miss any possible metastasis. We observed intense and overlapping VEGFR2 and PyMT specific IF staining, confirming the suspected metastatic activity (fig. 17). The areas were relatively small with diameters of approximately 1 mm. We also observed several small (10-500 µm) regions with PyMT staining that lacked the increase in VEGFR2 expression. Single cells with positive PyMT staining were also present, especially at the outer parts of the lungs (fig. 17).

Presumably, the larger metastasis had undergone an angiogenic switch and started to develop new vessels as indicated by the intense VEGFR2 staining. We were however not able to get PI and FI from the same sections in any of the animals with intense lung uptake which would have proven that the tracer uptake correlated to VEGFR2 expression also in these observations.
Figure 17. IF of lung tissue from one of the MMTV-PyMT mice in paper IV. To the left: areas with PyMT specific staining (green), ~100µm in diameter, are indicated with the white arrow and single cells with PyMT staining are indicated with yellow arrows. The VEGFR2 staining (red) is comparable to normal lung tissue in and around the PyMT stained area. The right image represents a metastases, ~1 mm in diameter, with intense VEGFR2 and PyMT staining. The same lung displayed high focal uptake of (R)-[^11]C]PAQ but not [^18]F]FDG as described in paper IV.

After (R)-[^11]C]PAQ PET a few mice were euthanized and lungs removed for PI and IF analysis. In the lungs that were removed and fixated with embedding medium (OCT)/phosphate buffered saline, to resemble an inflated lung, metastasis with marked VEGFR2 expression were unfortunately not detected. This was however in accordance with the normal uptake patterns of (R)-[^11]C]PAQ observed in these animals. In addition, some animals were injected with (R)-[^11]C]PAQ outside the camera and euthanized 30 min post injection with the intention to obtain better PI results due to higher radioactivity levels. This proved to be a good strategy, which produced the best PI images of the primary tumors and let us increase the amount of information obtained from each radiosynthesis.

The MRI scans of the euthanized animals added valuable morphological information, especially when locating the radioactivity uptake in the lungs and aiding in the comparisons of (R)-[^11]C]PAQ and [^18]F]FDG uptake patterns. Some of the animals displayed an uptake of (R)-[^11]C]PAQ in brown adipose tissue in the neck, which can in some cases be hard to delineate from lung tissue without appropriate morphological landmarks. More accurate landmarking for radioactivity uptake would probably have been obtained if the MRI was performed on live animals since a slight collapse of the body occurs after euthanasia, especially of the lung region. Further quantification improvements might also have been achieved if gating correction for respiratory motion had been applied for the PET data^{219}.

In summary, these results further demonstrate the promising angiogenesis imaging abilities of[^11]C]PAQ. By using a combination of different techniques we were able to obtain important information from a relatively small number of animals. Based on these results, we will hopefully be able to perform further preclinical studies with (R)-[^11]C]PAQ as a step towards a possible clinical implementation.
FUTURE DIRECTIONS

The pharmacokinetics, including the metabolism, of TKIs are often well studied during the developmental phase of the drug, but has only rarely been studied or considered in the evaluation of the corresponding PET tracers and derivatives. Several TKI-based PET tracers have been unsuccessful in imaging RTKs in vivo despite excellent in vitro properties. In some cases, this is likely caused by an extensive metabolism rendering them incapable of binding to the intended targets. The need for proper metabolism assessments is exemplified by our observations with $^{[11]}$C]PD153035 presented in this thesis. The extensive metabolism, indicated to be present also in humans, make the molecule very unreliable as a PET tracer since variations in parent drug to metabolite ratios can be highly individual, depending on metabolic capabilities, and the location of the lesion will play an crucial role for the uptake of the different radioactive components. Therefore, assuming a similar metabolism in humans, $^{[11]}$C]PD153035 is likely not suitable for patient stratification for EGFR-targeted therapy on a clinical basis.

$^{[11]}$C]PAQ and particularly the $R$-isomer have shown very interesting results in these studies, with good VEGFR2 detection capabilities in primary tumor tissue and, as indicated, most likely in VEGFR2 expressing pulmonary metastasis. It is, however, necessary to further investigate its use in cancer development monitoring and its abilities to quantify VEGFR2 expression in growing lesions with local angiogenic hotspots. This may be explored in preclinical longitudinal studies using spontaneous cancer models such as the MMTV-PyMT. Since this, and similar models, allow studies of several stages of disease, it also provides an excellent opportunity to study the ability of the tracer to detect differences in tumor characteristics as a response to treatment. Furthermore, it is necessary to investigate binding of the tracer to non-VEGFR2 targets such as the ABC-transporters and possibly other RTKs. Combined ex vivo analyses, such as IF and PI, of relevant tissue can be performed in order to correlate uptake to expression levels of a wider range of targets than those investigated in this thesis. It would also be possible to conduct in vitro assays to estimate specific interactions between $^{[11]}$C]PAQ and non-VEGFR2 targets. Pending positive results in these studies, subsequent micro-dosing studies in human cancer patients would be the ultimate challenge for the tracer. Hopefully, as one of very few TKI based PET tracers, $^{[11]}$C]PAQ can prove to be a valuable addition in cancer diagnostics, monitoring of disease progression and therapy response and aiding in anti-cancer drug development.
ACKNOWLEDGEMENTS

The following persons have all been very important during my time as a Ph.D. student and are gratefully acknowledged:

First of all, Sharon Stone-Elander, my main supervisor and guide in science. I thank you for inspiring me with your curiosity and endless optimism - always when it was most needed. Especially, I would like to thank you for believing in my ideas and for letting me take the time to finish the studies in my own way.

Jan-Olov “J-O” Thorell for sharing his fantastic laboratory skills and great knowledge in radiochemistry and for all support that made the chemistry in the projects work so well. I also thank you for the joyful fishing trips at svartsö, for showing me around söder occasionally and for “finding” me on the Friday afternoon bus 10 years ago.

Li Lu, one of the most important persons in my projects. Without you not a single preclinical experiment would have been possible. I thank you for always taking care of the “bananas” and for performing every experiment with such great skills and focus.

My co-supervisors Lars Holmgren and Stig Linder for all the great ideas and support and for introducing me to the right people. The use of your laboratory resources have been invaluable in my projects. A special thanks to Maria Hägg in Stigs group for all the help with IHC, cell culturing and general problem solving.

The current colleagues in the radiochemistry group at the neuroradiology department: Emma Jussing, Rebecka Dahlqvist, Anna Lindström and Oscar Hällqvist for being such great colleagues and friends, for always keeping the labs in order and for covering for me during my experiments and thesis writing.

My research colleagues Jonas Grafström and Tetyana “Tanya” Tegnebratt for taking your time to discuss scientific matters with me in a very constructive way. Both of you are true scientists and I enjoy being in the same research group as you.

Fabian Arnberg and Staffan Holmin for opening new doors in research during the very creative experiments we have done together. I hope for more beautiful collaborations in the future.

I thank Anna Freij for making me interested in the radiopharmaceutical science and for a great time during my first years at the pharmacy. You are a true fighter with a kind heart.

Birgit Garmelius for teaching me the basic principles of radiopharmaceutical production, which I will always will carry with me.
The staff of the old research department at the pharmacy; **Staffan Eksborg** for sharing his great knowledge in pharmacokinetics and for valuable support during the process of becoming a Ph.D. **Hans Ehrsson, Inger Wallin, Pernilla Videhult Pierre, Elin Jerremalm, Helene Nygren, Kjell Rudaeus** and **Carina Ritzmo**, for being such nice and welcoming colleagues when I first came to the pharmacy.

All personnel from the **KI-psychiatry** and **Astra Zeneca PET groups** for a smooth cooperation in the labs and for unforgettable memories during conference travels.

**Madjid Ebrahimi, Carsten Steiger** and **Hanna Hallenberg** for taking care of the PET radiochemistry facilities and for maintaining the good quality of the radiopharmaceutical productions – always in a very professionally way.

I would like to thank all members of the **Ulf Eriksson** research group at KI, particularly **Annika Melhelm** and **Carolina Hagberg**, for very interesting collaborations and excellent co-publications.

My gratitude also go to the Sel-tag imaging project – especially **Hanna-Stina Martinsson Ahlzén, Qing Cheng, Helena Wållberg** and **Elias Arnér** for nice collaborations, well organized seminars, entertaining dinners and several publications together.

**Peter Damberg, Helene Rundqvist** and **Jan Mulder** for adding new dimensions to the research in paper IV. I hope we can continue the collaboration in the future.

The personnel at **KERIC** for keeping the place up and running so we could perform the experiments and for being so kind and helpful in all situations.

To the ones who helped with improving the text and layout and reducing the number of spelling errors in this book – thanks!

**Alla mina vänner som jag träffar aldeles för sällan; Annica och Marcus, John och Anna, Fredrik och Pyrola, John och Barbara** för ert stöd och för alla trevliga stunder tillsammans. **Martin och Tobbe**, tack för den fantastiska studietiden i Uppsala som la grunden för mitt forskande - det jag minns kommer jag aldrig att glömma.

Mina föräldrar, **Bengt och Barbro**, min bror **Daniel** och hans familj hemma i de värmeländska skogarna. Tack för de välbehövliga uppehållen från den stressiga varden och för att ni alltid tar emot oss med öppna armar när vi kommer och hälsar på. Framför allt vill jag tacka mina föräldrar för att ni alltid låtit mig göra det jag varit intresserad av och för att ni har gjort mig till den jag är.

Slutligen vill jag tacka min fantastiska fru **Maria** för att du visar förståelse och stöttar mig när det behövs som mest. Våra underbara barn, **Isak och Klara** tackar jag för att ni har satt saker i rätt perspektiv och gjort mig nyfiken på de små sakerna i livet - igen. **Jag älskar er.**
REPRINT PERMISSIONS

All previously published papers were reproduced with permission from the publisher according to the following statements:

**Paper I**


**Paper II**


**Paper III**


**Paper IV**

Submitted

**Figure 10**

Reprinted from *Cell*, 144(5), *Hanahan, D. and R. A. Weinberg*, Hallmarks of cancer: the next generation (Figure 6), 646-674. © (2011), with permission from Elsevier
REFERENCES


32. Saleem, A.S., Graham; Kenny, Laura M.; Huiban, Mickael; Waldman, Adam; Downie, Louise; Lau, Mike; S Murphy, Philip; Kozlowski, Kasia; Lewis, Yvonne; Woodley, Laura; Hill, Sam; Kamalakaran, Aruloly; Hirschberg, Sandra; Kaneko, Tomomi; Aboagye, Eric; Marini, Luca; Coomber, R. Charles Brain and tumor penetration of carbon-11–labeled lapatinib ([11C]Lap) in patients (pts) with HER2-overexpressing metastatic breast cancer (MBC). 2013 ASCO Annual Meeting 2013. http://meetinglibrary.asco.org/content/116813-132, (Last visited Dec. 2013).


52


