Preparation and Evaluation of Molecular Imaging Probes Targeting the Urokinase Plasminogen Activator System

By

Alyssa Vito, B.Sc.

A Thesis
Submitted to the School of Graduate Studies
In Partial Fulfillment of the Requirements
For the Degree
Master of Science

McMaster University
MASTER OF SCIENCE (2015) McMaster University

(Chemistry and Chemical Biology Department) Hamilton, Ontario

TITLE: Preparation and Evaluation of Molecular Imaging Probes Targeting the Urokinase Plasminogen Activator System

AUTHOR: Alyssa Vito, B.Sc. (Eastern Michigan University)

SUPERVISOR: Dr. John Valliant

NUMBER OF PAGES: xiv, 85
Abstract

The aim of this thesis was to develop a molecular imaging probe for the urokinase plasminogen activator (uPA) system, which has been shown to play a critical role in cancer metastasis, tumour aggressiveness and likelihood of progression. Two classes of small molecule inhibitors carrying isotopes of iodine were synthesized and evaluated using in vitro assays and in vivo studies. Lead compounds showed high affinity for the target with $K_i$ values in the low nanomolar range ($1b = 1.4 \text{ nM}$, $1e = 6.1 \text{ nM}$, $1g = 2.6 \text{ nM}$ and $2a = 2.1 \text{ nM}$). Biodistribution studies of the reversible compounds ($1b$, $1e$, $1g$) showed rapid clearance, accumulation in the gall bladder and intestines and little to no tumour uptake (<1 %ID/g). The irreversible inhibitor ($2a$) showed specificity for the target through SDS-PAGE and biodistribution studies. Analysis of the biodistribution pattern showed retention in the tumour over time reaching a maximum at 24 h post-injection of 1.95 %ID/g with tumour-to-blood ratio being 0.65 at 24 h, 1.13 at 48 h and 1.09 at 96 h post-injection.

A parallel strategy reported involved targeting the uPA receptor (uPAR) through the use of antibodies and bioorthogonal chemistry based on radiolabeled tetrazines and transcyclooctene (TCO) functionalized biomolecules. A new tetrazine synthon was developed that can be readily labeled with both $^{99m}\text{Tc}$ and $^{18}\text{F}$ where the products were produced in 75 and 31 % radiochemical yields. Stability studies showed the compounds are suitable for use in vivo. Biodistribution studies were carried out in CD1 mice and results showed that both probes had sufficient distribution patterns to warrant use in pre-targeting strategies. Their reactivity with TCO, including functionalized derivatives such
as TCO-anti-uPAR, was also demonstrated creating the means to develop PET and SPECT probes for imaging the urokinase system using a single prosthetic group.
Acknowledgements

I would like to start by acknowledging my supervisor Dr. John Valliant for imparting his wisdom, expertise and experience with me during my time in his lab. I appreciate being given this opportunity to work in such a high-level facility with access to cutting edge equipment and innovative research. Your dedication to professionalism and extremely high standards has instilled in me key concepts that I will continue to carry forward into all future endeavors. It has been a privilege to work underneath you and I look forward to watching your career and success continue to grow in years to come.

I would like to thank my additional two committee members – Dr. Fred Capretta and Dr. Paul Harrison. Your input, constructive criticism and support has allowed me to delve further into my research and has pushed me to grow and excel in areas where I was previously weak. Thank you for the time you put into my project and thesis and in helping me to become a better, more well-rounded scientist.

I would like to thank all the members of the Valliant Research Group for their support, input and collaborations to my thesis. In particular, I would like to thank Silvia Albu for passing on her knowledge to me and teaching me the core basics of radiochemistry. I would also like to give a special thanks to Nancy Janzen for all of her help and support with the biology in my project.

I would like to give many thanks to my family, whom have always been an important support network and encouraged me to pursue higher aspirations. Thank you to my parents for leading by example by always being the hardest-working people I know. Your hard work has allowed me to get to where I am today and I am forever grateful.
Thank you to my sisters for always supporting and encouraging me and listening to me throughout my many times of need. You have been a much needed sounding board throughout this journey.

My greatest thanks of all is to my husband and former lab colleague, whose support has been invaluable to me. You inspire me every day to get up and work a little bit harder and are always pushing me to strive for something greater. Having you in my life has allowed me to be a better and stronger scientist, and overall a better person.

To my amazing son Malik, you are the light of my life. Your birth instilled in me a sense of pride, dedication and determination I had never felt before. This thesis, and all of my future work, is for you.
### Table of Contents

**CHAPTER ONE: INTRODUCTION**

1.1. Cancer 1

1.2. Molecular Imaging 2

1.3. Radiolabeling and Radioisotopes 7

1.4. The Urokinase Plasminogen Activator System 8

1.5. Hypothesis and Objectives 17

**CHAPTER TWO: RADIOLABELING AND BIOLOGICAL SCREENING FOR SMALL MOLECULE INHIBITORS OF UPA**

2.1. Small Molecule Inhibitors of uPA 19

2.2. *In Vitro* Colourmetric Assay 21

2.3. Radiochemistry 24

2.4. Cell Binding Assay for Reversible Inhibitors 28

2.5. SDS-PAGE 30

2.6. Biodistribution Studies 32

2.7. Summary and Conclusions 39

2.8. Experimental 39

2.8.1. General 39

2.8.2. *In Vitro* Colourmetric Assay 40

2.8.3. General Radiolabeling Procedure for Reversible Inhibitors 41

2.8.4. General Radiolabeling Procedure for Irreversible Inhibitors 42

2.8.5. Cell Binding Assay 43
2.8.6. SDS-PAGE 44

2.8.7. Biodistribution Studies 45

CHAPTER THREE: BIOORTHOGONAL CHEMISTRY and PRE-TARGETING STRATEGIES FOR IMAGING UPAR 47

3.0. Introduction 47

3.1. Pre-targeting and Bioorthogonal Chemistry 47

3.2. Radiolabeled Tetrazines 49

3.3. Hydrazinonicotinic Acid 51

3.4. Objectives 53

3.5. Chemical Synthesis 53

3.6. Radiolabeling with $^{99m}$Tc 54

3.7. Radiolabeling with $^{18}$F 56

3.8. Stability Studies 58

3.9. Reaction with (E)-Cyclooct-4-enol (TCO) 60

3.10. TCO Anti-uPAR Antibody Conjugation 62

3.11. Radio-TLC 64

3.12. Biodistribution Studies 65

3.13. Summary and Future Work 67

3.14. Experimental 68

3.14.1. General 68

3.14.2. Synthetic Procedures 69

3.14.3. General Radiolabeling Procedure for $^{99m}$Tc 77
3.14.4. General Radiolabeling Procedure for $^{18}$F

3.14.5. TCO Anti-uPAR Antibody Conjugation

3.14.6. Radio-TLC

3.14.7. Biodistribution Studies

REFERENCES
List of Figures

Figure 1 Annihilation reaction forming the basis of PET scans. ........................................... 4
Figure 2 Mammogram (left) and molecular breast imaging scan (right) of the same patient. Twenty-millimeter cancer can be seen on both images, but only the molecular breast image (using $^{99m}$Tc sestamibi) shows an additional 10 mm lesion (right). (Reprinted with permission of $^{20}$) ......................................................................................... 7
Figure 3 Representation of the proteolytic cascade initiated by uPA/uPAR and the resulting degradation of extracellular matrix. (Reprinted with permission of $^{31}$) ................. 10
Figure 4 Structures of the diaryl phosphonate inhibitors reported by Joossens et al. (“R” in compound 1 is equal to a, b, c, …). (Reprinted with permission of $^{46}$) ......................... 15
Figure 5 General structure for reversible inhibitors, 1a-1h. (“R2” in compound 1 is equal to a, b, c, …) ..................................................................................................................... 20
Figure 6 Structure of iodinated diphenyl phosphonate peptidic irreversible inhibitor, 2a. ........................................................................................................................................ 21
Figure 7 Representative plot of slope (where slope is equal to the ratio of the rate of the enzymatic reaction for a given concentration of inhibitor to the rate of the enzymatic reaction for no inhibitor) versus concentration of 1a for the determination of the $IC_{50}$ value ........................................................................................................................................ 23
Figure 8 HPLC analysis of the co-injection of 4b with the reference standard 1b. The similar retention time of 12 minutes confirms the identity of the radiolabeled compound. The top spectrum is the gamma chromatogram and the bottom spectrum is the UV-HPLC chromatogram ($\lambda = 254$ nm). Note that the detectors are connected in series (HPLC method B) ......................................................................................................................... 26
Figure 9 HPLC analysis of the co-injection of 4e with the reference standard 1e. Similar retention time of 11 minutes confirms the identity of the radiolabeled compound. The top spectrum is the gamma chromatogram and the bottom spectrum is the UV-HPLC chromatogram ($\lambda = 254$nm). Note that the detectors are connected in series (HPLC method A) ........................................................................................................................................ 26
Figure 10 HPLC analysis of the co-injection of 4g with the reference standard 1g. Similar retention time of 8.5 minutes confirms the identity of the radiolabeled compound. The top spectrum is the gamma chromatogram and the bottom spectrum is the UV-HPLC chromatogram. ($\lambda = 254$nm). Note that the detectors are connected in series (HPLC method A) ........................................................................................................................................ 26
Figure 11 HPLC of the co-injection of 6 with the reference standard 2a. Similar retention times of 15.5 minutes confirms the identity of the radiolabeled compound. The top spectrum is the gamma chromatogram and the bottom spectrum is the UV-HPLC
Alyssa Vito – M.Sc. Thesis; Chemical Biology – McMaster University

chromatogram. (λ = 254nm). Note that the detectors are connected in series (HPLC method A). ................................................................. 28

Figure 12 Cell assay results for 4b and MDA-MB-435, MDA-MB-231 and HT-1080 cells. All conditions were repeated in wells containing no cells as a control. (Group 1 contains cells and probe alone. Group 2 contains cells, probe and is acid washed after incubation. Group 3 contains cells, probe and is acid washed prior to incubation. Group 4 contains cells, probe and recombinant uPA and is acid washed prior to incubation)........ 29

Figure 13 SDS-PAGE analysis of 6 binding to HMW uPA. The intense band just below 37 kDa signifies a band for the complex between LMW uPA and 6. ........................................ 30

Figure 14 SDS-PAGE analysis of 6 binding to HMW-uPA, with 2b and PAI-1 as blocking agents. Lanes with no 2-mercaptoethanol clearly only show bands indicative of a complex formed with HMW-uPA, whereas those with 2-mercaptoethanol show bands indicative of a complex formed with LMW-uPA. 2b is a known and potent inhibitor of uPA, with complete inhibition seen. PAI-1 shows partial inhibition of the binding of 6. 32

Figure 15 Comparative graphical analysis of percent injected dose per gram, per organ/tissue harvested for 4b, 4e and 4g, all at 0.5 h PI in HT-1080 tumour xenograft model................................................................. 33

Figure 16 Graphical analysis of percent injected dose per gram, per organ/tissue harvested for 4b in both MDA-MB-231 and HT-1080 tumour xenograft models, 0.5 h and 2 h PI. ......................................................................................... 34

Figure 17 Percent injected dose per gram for 4b at 0.5 h and 2 h PI in HT-1080 and HT-29 tumour xenograft models (n = 3). Groups with uPA are indicative of groups that were pre-incubated with endogenous uPA for 0.5 h at 37 °C prior to injection........ 35

Figure 18 Percent injected dose per gram for 6 at 0.5 h, 2 h, 24 h, 48 h and 96 h PI in HT-1080 tumour xenograft models (n = 3 per time point)......................................................... 36

Figure 19 Blood clearance and tumor uptake of 6 in HT-1080 tumour xenograft model over 96 h. ................................................................. 37

Figure 20 Percent injected dose per gram for 6 with and without block at 48 h PI in HT-1080 tumour xenograft model (n = 3). .......................................................................... 38

Figure 21 Blood clearance and tumor uptake of 6 with and without block in HT-1080 tumour xenograft model 48 h PI. ................................................................. 38

Figure 22 Structure of Bio-Phen CS-61................................................................. 41

Figure 23 General scheme of tumour pre-targeting followed by antibody labeling with a radiolabeled tetrazine via an inverse-electron-demand Diels-Alder reaction. (Reprinted with permission of 62)................................................................. 48

Figure 24 γ-HPLC chromatogram of 5 following purification by HPLC (HPLC method A). ........................................................................ 55
Figure 25 Radiochemical yield of 5 versus the amount of 4 used during the initial labeling step. Note: The amount of $^{99m}$TcO$_4^-$ was consistent for each labeling experiment (740 MBq).

Figure 26 Radiochemical yield with respect to pH of the reaction.

Figure 27 HPLC analysis of the co-injection of 9 with the reference standard 11. The similar retention time of 15.5 minutes confirms the identity of the radiolabeled compound. The top spectrum is the UV-HPLC chromatogram and the bottom spectrum is the gamma chromatogram ($\lambda = 254$ nm). Note that the detectors are connected in series (HPLC method B).

Figure 28 $\gamma$-HPLC chromatograms of 5 in saline at 0.5 h, 1 h, 4 h and 6 h (top to bottom, HPLC method B).

Figure 29 $\gamma$-HPLC chromatograms of 11 in saline at 1 h, 1.5 h and 2 h (top to bottom, HPLC method B).

Figure 30 $\gamma$-HPLC chromatograms of the reaction between 5 and TCO (top) and 5 alone (bottom).

Figure 31 $\gamma$-HPLC chromatograms of the reaction between 9 and TCO (top) and 9 alone (bottom).

Figure 32 MALDI-TOF analysis. The top spectrum is for the uPAR antibody alone. Second spectrum is for the antibody +10 equivalents of (E)-cyclooct-4-enyl 2,5-dioxo-1-pyrrolidinyl carbonate. The third spectrum is for the uPAR antibody +20 equivalents of (E)-cyclooct-4-enyl 2,5-dioxo-1-pyrrolidinyl carbonate. Both the second and third spectrum show an average of 3.4 transcyclooctene conjugated per antibody.

Figure 33 Radio-TLC of compound 5. Note: Baseline of 50 mm used.

Figure 34 Radio-TLC of 5, conjugated with TCO-anti-uPAR antibody, as demonstrated by the new peak formed at 50 mm. Note: baseline of 50 mm used.

Figure 35 In vivo biodistribution of 5 in female CD1 mice at 0.5 h, 1 h, 2 h and 6 h PI. Data expressed as %ID/g.

Figure 36 In vivo biodistribution of 9 in female CD1 mice at 0.5 h, 1 h, 2 h and 4 h PI. Data expressed as %ID/g.

Figure 37 $^1$H NMR for compound 3.

Figure 38 $^{13}$C NMR for compound 3.

Figure 39 HRMS for compound 3.

Figure 40 $^1$H NMR for compound 4.

Figure 41 MS for compound 4.

Figure 42 $^1$H NMR for compound 7.

Figure 43 HRMS spectra for compound 11.
List of Tables

Table 1 Properties of the common radioisotopes of iodine. ............................................. 8
Table 2 Reversible inhibitor structures and affinities, as determined by colorimetric enzyme assay ................................................................. 22
Table 3 Irreversible inhibitor structures and affinities, as determined by a colorimetric enzyme assay ................................................................. 24
List of Schemes

Scheme 1 Radiolabeling of compounds 3b, 3e and 3g with $^{125}$I. a) 4b was radiolabeled with $^{125}$I and purified using a FSPE cartridge. The final compound was obtained in 99% purity in 25% radiochemical yield. b) 4e was radiolabeled with $^{125}$I and purified through semi-preparative HPLC. The final compound was obtained in 98% purity in 15% radiochemical yield. c) 4g was radiolabeled with $^{125}$I and purified through semi-preparative HPLC. The final compound was obtained in 99% in 20% radiochemical yield.

Scheme 2 Radiolabeling of 5 with $^{125}$I where the product was isolated using a silica column. The final compound (6) was obtained in 99% purity in 15% radiochemical yield. (R=butyl).

Scheme 3 Schematic of a modular strategy for the construction of $^{89}$Zr- and $^{64}$Cu-modified antibody bioconjugates using the tetrazine-norbornene ligation. (Reprinted with permission of 69).

Scheme 4 Reaction scheme for radiolabeling of HYNIC peptides with $^{99m}$Tc using tricine as coligand. (Reprinted with permission of 77).

Scheme 5 Synthesis of 4. Note: Compound 1 provided by Dr. Hosen Alarabi.

Scheme 6 Reaction scheme for the radiolabeling of compound 4 with $^{99m}$Tc.

Scheme 7 Synthesis of 7 for $^{18}$F labeling.

Scheme 8 Radiolabeling of 4 with $^{18}$F.

Scheme 9 Synthesis of non-radioactive reference standard, compound 11.

Scheme 10 Schematic for the conjugation of anti-uPAR antibody with (E)-cyclooct-4-enyl 2,5-dioxo-1-pyrrolidinyl carbonate.
### List of Abbreviations and Symbols

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D</td>
<td></td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td></td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>Arg</td>
<td></td>
<td>Arginine</td>
</tr>
<tr>
<td>Asp</td>
<td></td>
<td>Aspartate</td>
</tr>
<tr>
<td>β</td>
<td></td>
<td>Beta</td>
</tr>
<tr>
<td>²¹³Bi</td>
<td></td>
<td>Bismuth-213</td>
</tr>
<tr>
<td>¹¹C</td>
<td></td>
<td>Carbon-11</td>
</tr>
<tr>
<td>CT</td>
<td></td>
<td>Computed tomography</td>
</tr>
<tr>
<td>⁶⁴Cu</td>
<td></td>
<td>Copper-64</td>
</tr>
<tr>
<td>DCM</td>
<td></td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DFO</td>
<td></td>
<td>Desferrioxamine</td>
</tr>
<tr>
<td>DIPEA</td>
<td></td>
<td>Diisopropylethylamine</td>
</tr>
<tr>
<td>DMF</td>
<td></td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td></td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DOTA</td>
<td></td>
<td>1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid</td>
</tr>
<tr>
<td>¹⁸F</td>
<td></td>
<td>Fluorine-18</td>
</tr>
<tr>
<td>FDG</td>
<td></td>
<td>Fluorodeoxyglucose</td>
</tr>
<tr>
<td>fpVCT</td>
<td></td>
<td>Flat-panel volume computed tomography</td>
</tr>
<tr>
<td>γ</td>
<td></td>
<td>Gamma</td>
</tr>
<tr>
<td>h</td>
<td></td>
<td>Hour</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>Water</td>
</tr>
<tr>
<td>HCl</td>
<td></td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HPLC</td>
<td></td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HRMS</td>
<td></td>
<td>High resolution mass spectrometry</td>
</tr>
<tr>
<td>HYNIC</td>
<td></td>
<td>Hydrazinonicotinic acid</td>
</tr>
<tr>
<td>Hz</td>
<td></td>
<td>Hertz</td>
</tr>
<tr>
<td>ID/g</td>
<td></td>
<td>Injected dose per gram</td>
</tr>
<tr>
<td>Ile</td>
<td></td>
<td>Isoleucine</td>
</tr>
<tr>
<td>¹¹¹In</td>
<td></td>
<td>Indium-111</td>
</tr>
<tr>
<td>¹²³I</td>
<td></td>
<td>Iodine-123</td>
</tr>
<tr>
<td>¹²⁴I</td>
<td></td>
<td>Iodine-124</td>
</tr>
<tr>
<td>¹²⁵I</td>
<td></td>
<td>Iodine-125</td>
</tr>
<tr>
<td>¹³¹I</td>
<td></td>
<td>Iodine-131</td>
</tr>
<tr>
<td>J</td>
<td></td>
<td>Coupling constants</td>
</tr>
<tr>
<td>kDa</td>
<td></td>
<td>KiloDalton</td>
</tr>
<tr>
<td>keV</td>
<td></td>
<td>Kiloelectronvolt</td>
</tr>
<tr>
<td>Lys</td>
<td></td>
<td>Lysine</td>
</tr>
<tr>
<td>M</td>
<td></td>
<td>Molar</td>
</tr>
<tr>
<td>MALDI-TOFMS</td>
<td></td>
<td>Matrix-assisted laser desorption/ionization time of flight mass spectrometry</td>
</tr>
<tr>
<td>MBq</td>
<td></td>
<td>Mega Becquerel</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>MeV</td>
<td>Megaelectronvolt</td>
<td></td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
<td></td>
</tr>
<tr>
<td>MHz</td>
<td>Megahertz</td>
<td></td>
</tr>
<tr>
<td>μL</td>
<td>Microliter</td>
<td></td>
</tr>
<tr>
<td>μmol</td>
<td>Micromole</td>
<td></td>
</tr>
<tr>
<td>MI</td>
<td>Molecular imaging</td>
<td></td>
</tr>
<tr>
<td>MIBI</td>
<td>Methoxyisobutylisonitrile</td>
<td></td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
<td></td>
</tr>
<tr>
<td>mmol</td>
<td>Millimole</td>
<td></td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
<td></td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
<td></td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
<td></td>
</tr>
<tr>
<td>NIRF</td>
<td>Near-infrared fluorescence</td>
<td></td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
<td></td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
<td></td>
</tr>
<tr>
<td>¹⁸O</td>
<td>Oxygen-18</td>
<td></td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
<td></td>
</tr>
<tr>
<td>PAI-2</td>
<td>Plasminogen activator inhibitor-2</td>
<td></td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
<td></td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
<td></td>
</tr>
<tr>
<td>PyBOP</td>
<td>Benzotria-zole-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate</td>
<td></td>
</tr>
<tr>
<td>RCY</td>
<td>Radiochemical yield</td>
<td></td>
</tr>
<tr>
<td>Rt</td>
<td>Retention time</td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
<td></td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
<td></td>
</tr>
<tr>
<td>SPECT</td>
<td>Single photon emission computed tomography</td>
<td></td>
</tr>
<tr>
<td>TCO</td>
<td>Transcyclooctene</td>
<td></td>
</tr>
<tr>
<td>⁹⁹ᵐTc</td>
<td>Technetium-99m</td>
<td></td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
<td></td>
</tr>
<tr>
<td>²⁰¹TI</td>
<td>Thallium-201</td>
<td></td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
<td></td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
<td></td>
</tr>
<tr>
<td>Tz</td>
<td>4-(1,2,4,5-tetrazin-3-yl)phenyl)methanamine</td>
<td></td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase plasminogen activator</td>
<td></td>
</tr>
<tr>
<td>uPAR</td>
<td>Urokinase plasminogen activator receptor</td>
<td></td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
<td></td>
</tr>
<tr>
<td>¹²⁴Xe</td>
<td>Xenon-124</td>
<td></td>
</tr>
<tr>
<td>¹²⁵Xe</td>
<td>Xenon-125</td>
<td></td>
</tr>
<tr>
<td>⁸⁹Zr</td>
<td>Zirconium-89</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER ONE: INTRODUCTION

1.1. Cancer

Cancer is a disease characterized by abnormal and uncontrolled cell growth. Although this typically begins in a localized region, it may grow and spread to other parts of the body causing systemic malignancy, or distant metastases. The word “cancer” itself is an umbrella term for over two hundred diseases that are all classified as subtypes of cancer. These subtypes, while varying in location, behavior, aggressiveness and many other factors, all share the commonalities of abnormal proliferation rates, high genetic mutation and potential for invasion into normal tissues.

In recent years, the field of oncology has seen an increased effort in the development and evaluation of new and improved diagnostic methods. The importance and significance of early diagnosis has been well established and studies have shown increased survival rates to be in direct correlation with early diagnosis. As well, the ability for a physician to obtain a more thorough and complete diagnosis in the early stages while utilizing quick, non-invasive procedures allows for not only better patient care, but also for more effective treatment strategies. Many diagnostic modalities for cancer to date come with limitations and even with a range of tests performed, doctors are still unable to determine key attributes accurately such as staging, aggressiveness and tumour margins without surgical intervention. Unfortunately, as a result many patients are undergoing unnecessary or suboptimal surgical and chemotherapy procedures. Having a diagnostic tool that could offer a non-invasive means to obtain similar
pathological results would change the course of treatment for many patients and enable better tailoring of oncologic treatment plans.

Molecular imaging (MI) is an area of rapid growth to help address the abovementioned limitations. MI techniques to date, while always rapidly evolving, still offer limited biochemical information and not all strategies work for all forms of cancer. Even when combined with other procedures (i.e.; blood work, needle biopsy, etc.) many times a physician is still unable to gather enough data for a complete diagnosis. The ability to develop new MI techniques for cancer diagnosis and characterization would not only offer more non-invasive means for patient care, but also give doctors the ability to deliver more precise and tailored treatment plans, sparing patients from side effects of unnecessary treatments.

1.2. Molecular Imaging

MI is a general terminology spanning methods for visualizing anatomical structures and biochemical targets and processes non-invasively.\(^2\) MI can also be used to highlight tumour margins and to guide surgical resection or focal therapies. The utilization of MI to its fullest potential requires the development and validation of probes that together with appropriate software and imaging devices can improve the management and treatment of diseases such as cancer.\(^2\)

Common types of anatomical imaging methods include computed tomography (CT), magnetic resonance imaging (MRI) and x-ray imaging; all of which provide a non-invasive means to observe anatomical structures as opposed to biochemical function. Nuclear imaging methods, such as positron emission tomography (PET) and single-
photon emission tomography (SPECT), are the most sensitive MI methods and give a distinct advantage over anatomical imaging tests as they are able to visualize pathophysiological, physiological and metabolic processes through the use of radiotracers. This is particularly advantageous in the field of oncology as diagnostic radiopharmaceuticals can provide insight into the unique biochemical aspects of tumours and they can be converted into therapeutic compounds by changing the nature of the isotope used. Compounds containing positron and gamma emitting isotopes are used for MI, while those containing beta and alpha emitters can be used for radionuclide therapy.\textsuperscript{3-5}

PET is based on the gamma ray emissions from compounds containing positron emitting radionuclides. PET is routinely used to measure sugar metabolism (glucose) and blood flow.\textsuperscript{6,7} In a PET scan, the radioactive tracer is administered by intravenous injection where emitted positrons undergo annihilation reactions with electrons and the resulting two co-linear 511 keV gamma rays are detected by the PET scanner (Figure 1). The PET camera detects and records the coincident arrival of all gamma ray pairs as the basis for reconstruction of dynamic PET scans\textsuperscript{8,9} so that the origin of the emission can be determined, creating a “physiological map” of the distribution of the agent.\textsuperscript{10} Fluorine-18 (\textsuperscript{18}F) is the most commonly used isotope for PET scans because it is readily produced on a cyclotron using a proton bombardment reaction and it emits low energy positrons that have a short path length \textit{in vivo}, increasing resolution of the image.
The most commonly used PET radiopharmaceutical in oncology is fluorodeoxyglucose (FDG). This agent works on the basis that tumour cells metabolize glucose at a greater rate than normal cells, allowing FDG to accumulate in tumour cells. Although FDG is extensively used in diagnostic testing for cancer, it is unable to predict the aggressiveness of tumours, which is critical in staging and planning of treatment for patients. It also poses the problem that it is non-specific and therefore may show false positives through uptake in non-cancer related conditions that have increased metabolic activity.\textsuperscript{11,12}

Another nuclear medicine imaging method used in oncology is technetium (\textsuperscript{99m}Tc) bone scans. In a bone scan, a \textsuperscript{99m}Tc phosphonate derivative concentrates in regions of high calcium turnover and a gamma camera detects the localization of the agent. Therefore, areas with high bone-rebuilding activity will show high radioactive uptake, thus serving as a good indicator of bone metastases. This, coupled with scanner accessibility and the fact that the technique utilizes a radiopharmaceutical containing the inexpensive radioisotope \textsuperscript{99m}Tc, makes bone scans a widely used form of MI for cancer. However, the sensitivity of standard bone scans which are performed on a standard...
gamma camera is typically limited to lesions >1 cm in size consequently in order to view smaller lesions SPECT instrumentation is typically required.\textsuperscript{13}

SPECT is a type of radioimaging method that measures the distribution of compounds containing gamma emitting radionuclides. SPECT cameras typically contain multiple scintillation crystals connected to multiple-photomultiplier tubes that are able to detect gamma rays. The SPECT camera head fitted with a collimator is positioned at multiple angles around the patient in an effort to accumulate as many views at specific angular intervals as possible. Multiple “counts” are obtained at each view and, in some cases multi-headed cameras are used to increase the speed of acquisition. Software allows the integration of the individual projections gathered from each head into a composite data set, which can then be re-displayed as tomographic slices.\textsuperscript{3,14} SPECT is most commonly used to visualize brain disorders and injuries, cardiac disease and bone disorders (such as cancer metastases).\textsuperscript{15}

SPECT imaging is more common than PET and in all countries the number of SPECT cameras far exceeds the number of PET cameras.\textsuperscript{16} The widespread use of SPECT is due in large part to radiopharmaceuticals derived from $^{99m}$Tc, which is inexpensive when compared to other isotopes. Other gamma emitting radionuclides used for SPECT include indium-111 ($^{111}$In), iodine-123 ($^{123}$I) and thallium-201 ($^{201}$TI). Although the equipment for SPECT is more inexpensive and readily available than PET imaging, its sensitivity is more than 100-fold lower than that of PET and the associated quantitation methods, which are often important in oncology, are not as well elaborated.\textsuperscript{17,18}
Outside of oncology one of the most commonly used radiopharmaceuticals is \( ^{99m}\text{Tc} \) Sestamibi. This organometallic complex consists of \( ^{99m}\text{Tc} \) bound to six methoxyisobutylisonitrile (MIBI) ligands. While mainly used for cardiac imaging, Sestamibi and related analogues are also used for molecular breast imaging studies. Results have shown early detection of breast cancer to be amongst the top predictors of overall survival of patients.\(^{19}\) However, current screening techniques for breast cancer are typically restricted to standard mammography procedures, which come with limitations. Mammography relies heavily on the density of breast tissue, which varies between patients and is heavily dependent on the age. For this reason, the sensitivity of mammography is low and tumour margins are rarely well delineated.\(^{16}\) Clinical research has shown that with standard mammography alone, primary tumours and/or secondary masses often go undetected. In a clinical study by Hruska et al. molecular breast imaging was able to detect additional disease not shown by standard mammography in 16% of breast cancer patients studied (Figure 2).\(^{20}\)
Figure 2 Mammogram (left) and molecular breast imaging scan (right) of the same patient. Twenty-millimeter cancer can be seen on both images, but only the molecular breast image (using $^{99m}$Tc sestamibi) shows an additional 10 mm lesion (right). (Reprinted with permission of $^{20}$).

1.3. Radiolabeling and Radioisotopes

A radionuclide is an atom with an unstable nucleus that has an excess of available energy, which is released through a process called radioactive decay. Radioactive decay is the emission of particles or photons of ionizing radiation from the unstable, high energy nucleus. There are numerous radionuclides but only a select number are suitable for use in nuclear medicine. The specific properties that lead to the selection of a radionuclide for PET or SPECT medical imaging include half-life, availability, cost and energy and type of emission.

While FDG-PET is a mainstay of tumour detection and characterization, compounds derived from iodine are gaining increasing attention because they can be used for PET and SPECT imaging and radiotherapy. There are thirty-seven isotopes of iodine,
but the most commonly used for research and nuclear medicine are $^{123}$I, $^{124}$I, $^{125}$I and $^{131}$I (Table 1). $^{125}$I is produced in nuclear reactors through neutron irradiation of high purity xenon-124 ($^{124}$Xe) gas, forming xenon-125 ($^{125}$Xe). This isotope then decays where the daughter radioisotope $^{125}$I is dissolved in high purity dilute sodium hydroxide solution. This approach allows for the production of $^{125}$I in large quantities in a cost effective manner. $^{125}$I is often used to iodinate new drug candidates to complete in vitro assays and biodistribution studies. However, the long half-life of $^{125}$I and low gamma ray emission energy makes it unsuitable as an isotope for MI studies in patients.

Table 1 Properties of the common radioisotopes of iodine.

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Half-Life</th>
<th>Type of Emission</th>
<th>Most Common Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{123}$I</td>
<td>13.2 hours</td>
<td>$\gamma$ (159 keV)</td>
<td>SPECT</td>
</tr>
<tr>
<td>$^{124}$I</td>
<td>4.18 days</td>
<td>$\gamma$ (2.14 MeV)</td>
<td>PET</td>
</tr>
<tr>
<td>$^{125}$I</td>
<td>60.1 days</td>
<td>$\gamma$ (35.5 keV)</td>
<td>Radiochemistry, In vitro assays, Auger therapy</td>
</tr>
<tr>
<td>$^{131}$I</td>
<td>8.07 days</td>
<td>$\beta$ (606 keV) $\gamma$ (364 keV)</td>
<td>Therapy, imaging</td>
</tr>
</tbody>
</table>

$^{18}$F, the most commonly used isotope for PET scans, is also a popular isotope of choice as it is readily available and has desirable radioactive properties. $^{18}$F is produced on a cyclotron through high energy proton bombardment of oxygen-18 ($^{18}$O). Its ideal emission of low energy positrons with a short path length in vivo, coupled with its short physical half-life of 110 minutes makes it a suitable radionuclide for MI studies.

1.4. The Urokinase Plasminogen Activator System

The urokinase plasminogen activator (uPA) system is composed of serine proteases (uPA, tPA), a glycolipid-anchored receptor (uPAR), protease inhibitors of the
serpin family (PAI-1, PAI-2) and various binding proteins. uPA is a serine protease of the trypsin family that plays an important, though not fully understood, role in cell adhesion, migration and proliferation.\textsuperscript{26} Endogenous uPA is a 53 kDa zymogen containing 411 amino acids. It may be secreted from endothelial cells, fibroblasts, macrophages, smooth muscle cells, tumour cells or adjacent stroma.\textsuperscript{27} Upon binding to its receptor (uPAR), uPA is activated through cleavage of the Lys158-Ile159 bond. This produces an active, two-chain form of uPA, which is held together by a disulfide bond. Activated uPA then cleaves an Arg561-Val562 bond in a second zymogen, plasminogen, converting it into its active form, plasmin. This activation of plasmin triggers a proteolytic cascade that plays a key role in cellular invasiveness (Figure 3). This allows for tissue remodeling, which often results in any of a number of various diseases including arthritis, multiple sclerosis and tumour invasion and metastasis.\textsuperscript{28-30}
Figure 3 Representation of the proteolytic cascade initiated by uPA/uPAR and the resulting degradation of extracellular matrix. (Reprinted with permission of 31).

uPA contains three distinct binding domains. The N-terminal domain (residues 1-48), also known as the growth-factor domain, is the site responsible for uPA-uPAR binding and thus serves as a target for inhibitors of uPA. The second domain is the
kringle domain (residues 49-135). The third domain is the C-terminal domain (residues 136-411), also known as the serine protease domain, and is responsible for the activation of plasminogen to plasmin, thus making it a target for serine protease inhibitors.\textsuperscript{32,33}

Research studies have shown elevated levels of uPA and uPAR to be an indicative marker of cancer, and in particular disease aggressiveness and likelihood of progression. For this reason, the uPA system has become an attractive target for both the development of new molecular imaging probes as well as the synthesis of new anticancer drugs.\textsuperscript{32,34,35}

Clinical studies have shown uPA to be up-regulated in and surrounding tumour cells. In a study of 8,377 breast cancer patients, uPA and its endogenous inhibitor, plasminogen activator inhibitor-1 (PAI-1), were found to be key prognostic markers of disease free survival next to lymph-node status. In patients with lymph-node negative status, increased levels of uPA/PAI-1 were strong predictors of metastasis and therefore were found to be especially useful in designing treatment strategies.\textsuperscript{19}

In a clinical study of thirty patients, pathogenesis of human pancreatic cancer was studied and uPA and uPAR levels were quantified by northern blot analysis. Results showed a six-fold increase in the level of uPA and a four-fold increase in the level of uPAR in the pancreatic carcinoma tissue, when compared to normal, non-malignant pancreatic tissue samples. Upon correlation of these findings with the clinical parameters of the patients, it was indicative that patients with concomitant overexpression of uPA and uPAR had a shorter post-operative survival. This data suggests that uPA and uPAR can be used as prognostic markers in human pancreatic cancer.\textsuperscript{36}
Due to the properties of the uPA system and its up-regulation in tumour cells, it is an ideal target for molecular imaging. The ability to image the amount of uPA present in and surrounding a tumour would allow for clear visualization of the primary tumour, metastases and assessing aggressiveness. The ability to obtain such information in a non-invasive manner, prior to needle biopsy or surgical intervention, would allow for more specific, tailored treatment plans, sparing patients from unnecessary or incorrect interventions.37

Many different methods have been taken to synthesize molecular imaging probes targeting uPA. In one approach recombinant human plasminogen activator inhibitor-2 (PAI-2) was radiolabeled with $^{125}$I. Biodistribution studies were conducted on uPA-positive HCT-116 cells (colorectal carcinoma cells), with no significant tumour uptake (<1%). In another study, the inhibitory property of PAI-2 was again studied by radiolabeling it with the cytotoxin bismuth-213 ($^{213}$Bi), an alpha-emitting radioisotope. This study showed inhibitory capabilities of alpha-PAI-2 and it also showed no cytotoxic effects to freshly isolated normal human leukocytes, confirming that cells which do not contain active, receptor bound uPA cannot be targeted by alpha-PAI-2. It was suggested that this system can be used as a novel therapeutic agent for breast cancer, although no in vivo testing has been conducted to date.37–39

Weissleider and co-workers developed a fluorescent imaging probe for uPA with a peptide sequence mimicking that of the endogenous zymogen, plasminogen. The probe, which is recognized by uPA and cleaved between arginine and serine residues, has a low fluorescent signal in its initial state, but becomes strongly fluorescent after uPA
proteolysis. Near-infrared fluorescence images taken 6 hours and 24 hours after probe injection demonstrated high fluorescent signals using HT-1080 (fibrosarcoma cells) and HT-29 (adenocarcinoma cells) in vivo tumour models. However, optical imaging methods are limited by their poor depth of penetration (~3-5 mm). Thus, there remains a need and an opportunity for development of a PET or SPECT probe for imaging uPA.33

One of the challenges faced with when developing a molecular imaging probe targeting uPA is that uPA itself is a freely circulating protein, naturally found in the blood. However, literature has shown uPA to be localized in much higher concentrations at and around the site of a tumour, providing a route to achieving contrast so long has radiopharmaceuticals with high affinity for the protein can be developed.27,28,40 Due to the nature of uPA and its three distinct binding sites, there are many different approaches that can be used to target the protein in vivo. One approach described in the literature involves the use of antibodies to disrupt the uPA-uPAR binding.41 Alternatively, small molecule inhibitors have been developed to inhibit uPA which can also be used to develop MI probes.40,41

One class of reversible inhibitors that have shown good selectivity for uPA were derived from phenylguanidines. Various substituted phenylguanidines are reported in the literature, with Ki values in the micromolar range. Although most do not have very high binding affinity, they have been shown to have good selectively for the protein. In contrast, the amidinophenylalanine-type uPA inhibitor (WX-UK1) and its pro-drug, developed by Wilex company, has an affinity for uPA with a reported Ki value of 0.41...
nM. Despite being in phase I/II clinical trials, the specificity of this compound for uPA over other proteases is moderate.\textsuperscript{32,42,43}

An alternative class of small molecule inhibitors that are attractive platforms for developing a uPA probe are based on a series of peptides containing amidines. The amidine-based, peptide-derived class of inhibitors were designed to interact with an aspartate residue (Asp189) in the uPA active site that is involved in the interaction with an arginine residue (Arg560) on plasminogen. The peptide and its guanidine derivative show inhibitory activities of uPA in the low nanomolar range.\textsuperscript{44}

For irreversible inhibitors, a series of small nonpeptidic diaryl phosphonate inhibitors reported by Joossens \textit{et al.} (Figure 4) were identified as potential probe candidates. They reported compounds with IC\textsubscript{50} values in the low nanomolar range and more than 1000-fold selectivity over other highly related proteases. Further \textit{in vivo} screening with two of their lead compounds showed promising antimetastatic properties in the BN-472 rat mammary carcinoma model, with no signs of acute toxicity.\textsuperscript{45} These compounds like the reversible peptides described above provide a good starting basis for creating uPA targeted radiopharmaceuticals.
Figure 4 Structures of the diaryl phosphonate inhibitors reported by Joossens et al. ("R" in compound 1 is equal to a, b, c, ...). (Reprinted with permission of 46).
An alternative to targeting uPA is to prepare a probe that can target uPAR instead. uPAR is a heterogeneously glycosylated, single-chain polypeptide of molecular weight of 50-60 kDa, which decreases to 35 kDa upon deglycosylation. Under normal physiological conditions, the cellular expression of uPAR is low and it has been shown to markedly increase under specific stress conditions including tissue remodeling, inflammation, and immune response.

Several uPAR targeted probes can be found in the literature spanning various imaging modalities including optical, MRI, nuclear and multi-modal probes. One example of an optical probe targeting uPAR implemented a semi-automated approach to correlate two-dimensional (2D) noninvasive near-infrared fluorescence (NIRF) imaging with three-dimensional (3D), high-resolution, flat-panel volume computed tomography (fpVCT). Anti-uPAR antibodies labeled with a fluorescent cyanine dye (Cy5.5) were used to visualize human mammary cancer MDA-MB-231 xenograft in mice and a strong correlation between tumour size and fluorescence intensity was observed. In hopes of utilizing the advantageous anatomical resolution of MRI, a recombinant peptide derived from mice targeting uPAR was conjugated to magnetic iron oxide nanoparticles. Although high and specific uptake in uPAR-expressing cells was observed, translation to humans is potentially problematic in that to achieve adequate contrast large quantities of the agent would need to be administered.

Nuclear imaging probes targeting uPAR for both SPECT and PET imaging have also been reported. One example is an $^{111}$In-labeled peptide that binds to uPAR which is designed as a potential probe for SPECT imaging. Initial enzymatic assay results show
the probe to have IC\textsubscript{50} values 300 fold higher than that of uPA itself, suggesting that endogenous uPA would compete with the labeled peptide for binding of uPAR. This inference was supported by the biodistribution studies and through comparison of \textit{ex vivo} and \textit{in vivo} data. Several attempts to develop a PET probe targeting uPAR have also been reported. For instance, a \textsuperscript{64}Cu-labeled high-affinity anti-uPAR peptide was evaluated in a mouse tumour model bearing uPAR-positive U87MG human glioblastoma xenograft. Biodistribution studies showed tumour uptake that was ten-fold higher than that in the negative cell line (MDA-MB-435 cells).\textsuperscript{53} In fact, this interesting high-affinity uPAR peptide antagonist probe (known as \textsuperscript{64}Cu-DOTA-AE105) was evaluated in a quantitative PET study,\textsuperscript{54} and is currently in human clinical trials.

LeBeau \textit{et al.} recently reported the use of two humanized anti-uPAR antagonistic antibodies for both \textit{in vitro} and \textit{in vivo} studies.\textsuperscript{55} \textit{In vitro} results indicated that both of these antibodies can bind efficiently to the surface of MDA-MB-231 uPAR expressing cells based on a fluorescence study. \textit{In vivo}, SPECT images and near-infrared optical images of appropriately labeled forms of their antibodies confirmed the ability to detect uPAR expression in triple negative breast cancer tumour xenografts. Furthermore, LeBeau \textit{et al.} were also able to demonstrate the ability to target uPAR with these antibodies for the purpose of imaging acquired drug resistance to varying forms of chemotherapy.\textsuperscript{56}

\textbf{1.5. Hypothesis and Objectives}

The uPA system and its role in cancer has been thoroughly explored in both laboratory and clinical settings. Although many attempts have been made in targeting
both uPA and uPAR through the use of small molecules and/or antibodies, to date there are no MI probes for either that are currently used for clinical studies. The core hypothesis is that the development of a MI probe for the uPA system can be achieved by radiolabeling potent reversible or irreversible inhibitors or through the use of antibody based vectors against uPAR. The ultimate objective is to find a radiolabeled compound that would allow for non-invasive assessment of tumour aggressiveness and metastatic potential.

This thesis investigates methods for the preparation of radiolabeled small molecule inhibitors of uPA including both the reversible and irreversible inhibitors (chapter 2). Due to problems encountered with targeting uPA itself, work transitioned from that of small molecule based inhibitors, to targeting uPAR through the use of antibodies (Chapter 3). More specifically, the focus was on developing a new synthon for use in pre-targeting strategies with functionalized antibodies.
CHAPTER TWO: RADIOLABELING AND EVALUATION OF SMALL MOLECULE INHIBITORS OF UPA

2.1. Small Molecule Inhibitors of uPA

In the work presented in this chapter, two classes of small molecule inhibitors were radiolabeled and screened as potential molecular imaging probes for uPA. The first class of compounds was a family of reversible inhibitors that are based on a dipeptide system described by Levy and coworkers and examined by Steinmetzer et al.\textsuperscript{57} Within this series, the most promising was the D-serine / L-alanine dipeptide capped at the N-terminus with a benzylsulfonamide and at the C-terminus with a 4-(aminomethyl)-phenylguanidine moiety (1, Figure 5), which had a reported \( K_i \) value of 2 nM. The ability for substitution of polar amino acids in place of serine or alanine within this system allows for the opportunity to fine tune the pharmacokinetic properties and potentially enhance the specificity for uPA over other proteases.
Figure 5 General structure for reversible inhibitors, 1a-1h. (“R2” in compound 1 is equal to a, b, c, …).

Structure-activity relationships for this class of inhibitors indicates that addition of an isotope at R1 would not have a major influence on the binding affinity of the inhibitor. For this work, we chose to use iodine, to allow for the potential creation of a dual-functioning probe for both imaging and radiotherapy. The approach taken was that labeled inhibitors containing a stable-isotope of iodine were synthesized first for in vitro screening. This was designed to assess the impact of the halogen on the affinity for uPA prior to preparing the radioactive analogue. Compounds retaining the desired binding affinity were subsequently converted into their radiolabeled analogues and in vivo biodistribution studies performed.

Due to the fact that uPA is a freely circulating protein found within the body, it was hypothesized that the use of inhibitors that are reversible in nature may not allow for localization and accumulation of probe at the desired site of interest. For this reason, a
second class of inhibitors was synthesized, which were irreversible in nature. Joossens et al. reported the first irreversible, selective, and potent diphenyl phosphonate peptidic inhibitors for uPA.\(^{58}\) They described not only the selectivity of these inhibitors for uPA, but also the low toxicity and potential benefits of having an irreversible-based inhibitor versus a reversible one which included increased selectivity for uPA and decreased mechanism-based toxicity.\(^{45}\) Building on this work, we developed an iodinated diphenyl phosphonate inhibitor as a potential molecular imaging probe for uPA (Figure 6, 2a) following the same approach used for the abovementioned reversible inhibitors.

![Figure 6 Structure of iodinated diphenyl phosphonate peptidic irreversible inhibitor, 2a.](image)

**2.2. In Vitro Colorimetric Assay**

Compounds \(1a - 1h\), which were prepared by Dr. Silvia Albu, were evaluated for their inhibitory activity against uPA using a colorimetric enzyme assay (Table 2). Each inhibitor, at various concentrations, was incubated with uPA and upon addition of the chromogenic substrate Bio-Phen CS-61 (44), the course of the hydrolysis reaction was monitored.
Table 2 Reversible inhibitor structures and affinities, as determined by colorimetric enzyme assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>K_i (nM)</th>
<th>95% CI</th>
<th>IC_50 (nM)</th>
<th>95% CI</th>
<th>r^2</th>
<th>Std Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>6.7</td>
<td>6.1 – 7.4</td>
<td>24</td>
<td>21 – 26</td>
<td>0.996</td>
<td>1.1</td>
</tr>
<tr>
<td>1b</td>
<td>1.4</td>
<td>1.2 – 1.6</td>
<td>4.7</td>
<td>4.1 – 5.6</td>
<td>0.989</td>
<td>1.1</td>
</tr>
<tr>
<td>1c</td>
<td>250</td>
<td>99 – 650</td>
<td>760</td>
<td>300 – 1900</td>
<td>0.939</td>
<td>2.6</td>
</tr>
<tr>
<td>1d</td>
<td>48</td>
<td>35 – 66</td>
<td>170</td>
<td>120 – 230</td>
<td>0.940</td>
<td>1.2</td>
</tr>
<tr>
<td>1e</td>
<td>6.1</td>
<td>5.1 – 7.3</td>
<td>21</td>
<td>18 – 26</td>
<td>0.987</td>
<td>1.1</td>
</tr>
<tr>
<td>1f</td>
<td>1.6</td>
<td>1.2 – 2.2</td>
<td>5.7</td>
<td>4.3 – 7.6</td>
<td>0.930</td>
<td>1.2</td>
</tr>
<tr>
<td>1g</td>
<td>2.6</td>
<td>2.3 – 2.9</td>
<td>9.1</td>
<td>8.1 – 10</td>
<td>0.990</td>
<td>1.1</td>
</tr>
<tr>
<td>1h</td>
<td>20</td>
<td>17 – 25</td>
<td>72</td>
<td>59 – 87</td>
<td>0.988</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Note: Colorimetric assay data for compounds 1a, 1c, 1d and 1f were determined by Dr. Silvia Albu.

Compound 1a was previously prepared and tested using enzyme activity assay by Zeslawska et al., who reported a K_i value of 2 nM. This compound was prepared and used to validate the assay where the observed K_i value was 6.7 nM which was in agreement with the literature data given the observed standard errors. The remainder of the compounds were tested and many of the iodinated derivatives were shown to have comparable in vitro activities with K_i values in the low nanomolar range. However, the
binding affinity decreased considerably for iodinated analogue 1c, which possesses the iodine moiety near the guanidine group. Substitution of the alanine at the P2 position with the tyrosine (compound 1d) also yielded a less potent derivative ($K_i = 47.9$ nM).

![Figure 7](image)

**Figure 7** Representative plot of slope (where slope is equal to the ratio of the rate of the enzymatic reaction for a given concentration of inhibitor to the rate of the enzymatic reaction for no inhibitor) versus concentration of 1a for the determination of the IC$_{50}$ value.

For the irreversible class of inhibitors, which were also prepared by Dr. Silvia Albu, the enzyme binding affinity for uPA was measured using the same colorimetric assay (Table 3), as described above. Compound 2b which has a reported a $K_i$ value of 1.7 nM was used as the positive control.$^{45}$ In our assay, the compound showed good agreement with the literature having a $K_i$ value of 1.7 nM. The iodinated derivative, 2a, was tested and shown to have comparable $K_i$ value of 2.09 nM.
Table 3 Irreversible inhibitor structures and affinities, as determined by a colorimetric enzyme assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ (nM)</th>
<th>95% CI</th>
<th>$IC_{50}$ (nM)</th>
<th>95% CI</th>
<th>$r^2$</th>
<th>Std Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>2.1</td>
<td>1.2 – 3.5</td>
<td>7.3</td>
<td>4.3 – 12</td>
<td>0.98</td>
<td>1.3</td>
</tr>
<tr>
<td>2b</td>
<td>1.7</td>
<td>0.95 – 2.8</td>
<td>5.9</td>
<td>3.3 – 10</td>
<td>0.97</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Initial screening results of both the reversible and irreversible classes of inhibitors were successful in identifying lead compounds in each class to proceed with developing radiolabeled analogues. Reversible inhibitors $1b$, $1e$ and $1g$ as well as irreversible inhibitor $2a$ were selected to move forward based upon their low $K_i$ values.

2.3. Radiochemistry

All compounds were radiolabeled with $^{125}$I via electrophilic aromatic substitution (Scheme 1). Analytical HPLC was used to assess the purity of the product and co-injection with the non-radioactive reference standard was performed to confirm the nature of the products (Figure 8 – 10).
Scheme 1 Radiolabeling of compounds 3b, 3e and 3g with $^{125}$I. a) 4b was radiolabeled with $^{125}$I and purified using a FSPE cartridge. The final compound was obtained in 99% purity in 25% radiochemical yield. b) 4e was radiolabeled with $^{125}$I and purified through semi-preparative HPLC. The final compound was obtained in 98% purity in 15% radiochemical yield. c) 4g was radiolabeled with $^{125}$I and purified through semi-preparative HPLC. The final compound was obtained in 99% in 20% radiochemical yield.
Figure 8 HPLC analysis of the co-injection of 4b with the reference standard 1b. The similar retention time of 12 minutes confirms the identity of the radiolabeled compound. The top spectrum is the gamma chromatogram and the bottom spectrum is the UV-HPLC chromatogram (λ = 254 nm). Note that the detectors are connected in series (HPLC method B).

Figure 9 HPLC analysis of the co-injection of 4e with the reference standard 1e. Similar retention time of 11 minutes confirms the identity of the radiolabeled compound. The top spectrum is the gamma chromatogram and the bottom spectrum is the UV-HPLC chromatogram (λ = 254 nm). Note that the detectors are connected in series (HPLC method A).
Figure 10 HPLC analysis of the co-injection of 4g with the reference standard 1g. Similar retention time of 8.5 minutes confirms the identity of the radiolabeled compound. The top spectrum is the gamma chromatogram and the bottom spectrum is the UV-HPLC chromatogram ($\lambda = 254$nm). Note that the detectors are connected in series (HPLC method A).

The lead compound from the irreversible class of inhibitors was radiolabeled with $^{125}$I via electrophilic aromatic substitution (Scheme 2). The purity of the product was assessed by analytical HPLC and co-injection with the non-radioactive reference standard was again used to confirm the nature of the product (Figure 11).

Scheme 2 Radiolabeling of 5 with $^{125}$I where the product was isolated using a silica column. The final compound (6) was obtained in 99% purity in 15% radiochemical yield. (R=butyl).
Figure 11 HPLC of the co-injection of 6 with the reference standard 2a. Similar retention times of 15.5 minutes confirms the identity of the radiolabeled compound. The top spectrum is the gamma chromatogram and the bottom spectrum is the UV-HPLC chromatogram. (λ = 254nm). Note that the detectors are connected in series (HPLC method A).

2.4. Cell Binding Assay for Reversible Inhibitors

Inhibitor 4b was assessed in vitro with a series of cell binding assays. The purpose of this experiment was to determine if the probe will bind uPA, the uPA/uPAR complex, and to assess the extent of internalization and the degree of non-specific binding. Different cell lines were used to assess the impact of diverse levels of protein expression and to assess the best choice for a tumor xenograft. After multiple attempts, which included removing cell bound uPA by acid washing, the assay did not show any significant results where binding was highly variable. This is likely due to non-specific binding interactions between the test compounds and the plate itself (Figure 12).
Given the inconclusive nature of the cell studies, all three radiolabeled reversible inhibitors were assessed \textit{in vivo} through biodistribution studies. This step was taken because obtaining conclusive results from the cell assay would have required extensive optimization and time. Additionally, many uPA-expressing cell lines exhibit different amounts of uPA \textit{in vitro} than \textit{in vivo} and therefore results from cell studies may not necessarily be representative of the \textit{in vivo} biodistribution which is described in section 2.6.\textsuperscript{33}
2.5. SDS-PAGE

After successful radiolabeling of 5, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was conducted to confirm specific binding between the inhibitor and uPA (Figure 13).

![SDS-PAGE analysis of 6 binding to HMW uPA. The intense band just below 37 kDa signifies a band for the complex between LMW uPA and 6.](image)

The gel was successful showing one major band that is indicative of a complex of uPA and 7 through the use of molecular weight reference standards. The appearance of double bands at higher concentrations of uPA was due to the fact that the 2-mercaptoethanol, a reducing agent added to further denature the proteins by reducing
disulfide linkages, is believed to have cleaved the recombinant HMW-uPA to LMW-uPA.

Following initial SDS-PAGE analysis, a blocking study was conducted on 6 to confirm specificity of binding. The gel was carried out under the same conditions described above, with the addition of PAI-1 (endogenous inhibitor of uPA) and 2b as blocking agent, prior to incubation on ice. The gel successfully showed that 6 binds to uPA (Figure 14). It was also clear that 2-mercaptoethanol does indeed cleave HMW-uPA to LMW-uPA, although this has no effect on the binding ability of the inhibitor. Two positive controls, 2b^{58} and PAI-1, were able to effectively block 6 from binding to uPA, when they are already bound to the protein. However, 2b proved to be a more potent and effective blocking agent than the endogenous PAI-1 (which still allowed for some 6 to bind).
2.6. Biodistribution Studies

*In vivo* biodistribution studies were performed on the three lead compounds from the reversible class of inhibitors and results presented as percent injected dose per gram (%ID/g) (Figure 15 and 16).
Figure 15 Comparative graphical analysis of percent injected dose per gram, per organ/tissue harvested for 4b, 4e and 4g, all at 0.5 h PI in HT-1080 tumour xenograft model.
Figure 16 Graphical analysis of percent injected dose per gram, per organ/tissue harvested for 4b in both MDA-MB-231 and HT-1080 tumour xenograft models, 0.5 h and 2 h PI.

Following analysis of these initial biodistribution studies, it was hypothesized that the problem at hand is that the probe may not be reaching the target (tumour), but instead may be binding to freely-circulating proteins and thus never reaching the uPA on the leading edges of the tumour. A second attempt was made at biodistribution studies with 4b, this time with a pre-incubation with recombinant uPA prior to injection. The tumour model was also switched from MDA-MB-231 tumours to HT-29 tumours, as HT-29 cells are reported to be high expressers of uPA in vivo. Analysis of this biodistribution study, again, showed no significant tumour uptake (<1%) in all groups, with clearance through the gall bladder (Figure 17).
Figure 17 Percent injected dose per gram for 4b at 0.5 h and 2 h PI in HT-1080 and HT-29 tumour xenograft models (n = 3). Groups with uPA are indicative of groups that were pre-incubated with endogenous uPA for 0.5 h at 37°C prior to injection.

Following the evaluation of the reversible inhibitors, compound 6 was assessed in vivo (Figure 18). Analysis of the biodistribution pattern showed retention in the tumour over time reaching a maximum at 24 h post-injection of 1.95% ID/g with tumour-to-blood ratio being 0.65 at 24 h, 1.13 at 48 h and 1.09 at 96 h post-injection (Figure 19).

These results are comparable to those seen in biodistribution studies for other small molecule-derived probes for uPA, which show modest tumour uptake and increasing tumour-to-blood ratios. In one study, recombinant plasminogen activator inhibitor-2 (PAI-2), was radiolabeled with 125I and biodistribution studies were performed in mice carrying a subcutaneous HCT-116 human colon cancer xenograft. The results of this study showed rapid uptake in the tumour, with peak retention 30 minutes post-
injection at 1.3 %ID/g. Although the majority of radioactivity localized to major organs (i.e.; liver and kidneys), radioactivity cleared from these organs much more rapidly when compared to tumour tissues. The study also showed that increasing the dose of radioactivity injected increased tumour uptake, while keeping uptake in other organs relatively unchanged.\textsuperscript{60}

\textbf{Figure 18} Percent injected dose per gram for 6 at 0.5 h, 2 h, 24 h, 48 h and 96 h PI in HT-1080 tumour xenograft models (n = 3 per time point).
To assess the specificity of \( \textbf{6} \), the compound was administered along with \( \textbf{2b} \) as a blocking agent (Figure 20). Analysis of the biodistribution pattern for \( \textbf{6} \) showed retention in the tumour over time (peaking at 24 h post-injection with 1.95 %ID/g) with tumour-to-blood ratios of 0.65 at 24 h, 1.13 at 48 h and 1.09 at 96 h post-injection (Figure 21). This data showed a decrease in all tissues with the exception of the kidneys and gall bladder, which increased by 70% and 38%, respectively. Tissues with a notable decrease in uptake when administered with the blocking agent include the blood, adipose, bone, brain, heart and skeletal muscle, which all saw a decrease of at least 60%. The highest decrease was seen in the tumour itself, with a decrease of 70% when administered the blocking agent. This significant decrease indicates that the tumour is likely an area of specific uPA binding.
Figure 20 Percent injected dose per gram for 6 with and without block at 48 h PI in HT-1080 tumour xenograft model (n = 3).

Figure 21 Blood clearance and tumor uptake of 6 with and without block in HT-1080 tumour xenograft model 48 h PI.
2.7. Summary and Conclusions

Three reversible and one irreversible compounds containing iodine were assayed for uPA binding. Multiple high affinity leads were identified where four radiolabeled analogues were synthesized in high purity and characterized by comparison to non-radioactive reference standards. *In vitro* and *in vivo* assays with the reversible inhibitors failed to show any significant uPA binding or tumor binding and were therefore not explored further. The irreversible inhibitor showed high affinity and selectivity for uPA, which was confirmed through various *in vitro* and *in vivo* assays. Biodistribution studies of the irreversible inhibitor showed increasing accumulation in the tumour over time with good target to non-target ratios.

The lack of tumour uptake with any of the high affinity compounds brings in to question the choice of uPA as the target. It may be that there is too much endogenous uPA to take advantage of the increased expression in the tumour microenvironment. For this reason, an alternative approach was explored that would enable antibodies to be used to target uPAR. This approach could use pre-targeting and bioorthogonal chemistry and involve the development of a new class of radiolabeled tetrazines (chapter 3).

2.8. Experimental

2.8.1. General

Unless otherwise stated, all chemical reagents were purchased and used as received from Sigma-Aldrich without further purification. Solvents were purchased from Caledon. Reverse phase analytical HPLC was performed using a Varian HPLC. HPLC grade water containing 0.1% trifluoroacetic acid (solvent A) and acetonitrile containing
0.1% trifluoroacetic acid (solvent B) were used as eluents. Analytical HPLC was performed using a C18 reverse phase Phenomenex column (25 × 4.60 mm, 5 micron) while semi-preparative HPLC was performed using a C18 reverse phase Phenomenex Gemini column (250 × 10.00 mm, 5 micron). Analytical HPLC method A had a flow rate of 1 mL/min and a 95 to 5% gradient (Solvent A: Solvent B) over 30 minutes. Analytical HPLC method B had a flow rate of 1 mL/min and a 95 to 5% gradient (Solvent A : Solvent B) over 20 minutes. Method C, which was used for semi-preparative HPLC had a flow rate of 4 mL/min and a 95 to 5% gradient (Solvent A : Solvent B) over 30 minutes.

2.8.2. In Vitro Colorimetric Assay

All compounds were screened with an in vitro colorimetric enzyme assay involving Bio-Phen CS-61 (44) substrate (Aniara, A2209061) (Figure 22). Each inhibitor, at various concentrations, was incubated on a 96-well microtitre plate with 50 µL of 695 nM stock of uPA (EMD Chemicals Millipore, 672081) for 15 min at 37 ºC. Upon addition of the substrate, the course of the hydrolysis reaction was monitored by detecting the formation of p-nitroaniline at 405 nm and 37 ºC over the course of 30 minutes. The reaction rates obtained in the presence of each compound were compared to those seen with substrate alone and the percent inhibition was calculated. Previously determined K_m and percent inhibition were used for K_i calculations. All assays were run in triplicate three times with the exception of 1c, which was only run once in triplicate.
2.8.3. General Radiolabeling Procedure for Reversible Inhibitors

Eppendorf vials were coated with Iodogen (1,3,4,6-tetrachloro-3R,6R-diphenylglycouril) by evaporating a solution of the oxidant in chloroform (10 μL, 1 mg/mL) under a stream of argon. To an Iodogen coated vial was added a solution of the trialkylstannyl precursor in 5% glacial acetic acid in methanol (80 μL, 5 mg/mL), and aqueous sodium [125I]iodide (20 μL, 29-44 MBq). The reaction mixture was allowed to stand for 25 minutes with occasional swirling then quenched with aqueous sodium metabisulfite (100 μL, 0.01 mol/L). Compounds containing the fluorous tin precursor (3b) were then loaded onto a fluorous solid phase extraction (FSPE) cartridge that had been conditioned with DMF (5 mL) and washed with water (10 mL). The reaction vial was rinsed with methanol (100 μL), which was added to the FSPE cartridge. The cartridge was then eluted with water (10 mL) followed by 80% MeOH-water (~80 mL, collected in 20 mL fractions). The respective activities of the collected fractions were measured using a dose calibrator. HPLC method A was used for assessing the purity. Reactions involving trimethyl tin precursors 3e and 3g were purified via semi-preparative HPLC method C. Radioactive compounds were concentrated to dryness and TFA (2 mL) added and the reaction mixture was left to stand for 4 hr, before being concentrated under
a stream of argon. Analytical HPLC method B was used to assess the purity of the product and co-injection with the non-radioactive reference standard was obtained to confirm results of 4b and analytical HPLC method A was used for 4e and 4g.

2.8.4. General Radiolabeling Procedure for Reversible Inhibitors

A solution of Iodogen (1,3,4,6-tetrachloro-3R,6R-diphenylglycouril) was concentrated in acetonitrile (1 mg/mL). To an Eppendorf vial was added a solution of the trialkylstannyl precursor in 5% glacial acetic acid in acetonitrile (80 μL, 5 mg/mL), and aqueous sodium [125I]iodide (40 μL, 58-88 MBq). The reaction mixture was allowed to stand for 6 minutes with occasional swirling then quenched with aqueous sodium metabisulfite (100 μL, 0.01 mol/L). The reaction mixture was concentrated to remove excess acetonitrile under a stream of air. The reaction mixture was then loaded onto a silica column that had been conditioned with hexanes (10 mL). The reaction vial was rinsed with hexanes (100 μL), which was added to the silica column. The cartridge was then eluted with a gradient of hexanes : ether (20 mL) starting with pure hexanes where the product eluted with 2:3 v/v hexanes : ether. The respective activities of the collected fractions were measured using a dose calibrator. HPLC method A was used for assessing the purity. The desired compound (6) eluted with 40 hexanes : 60 ether. The product was concentrated to dryness and TFA (2 mL) was added and the reaction mixture was left to stand for 4 h, before being concentrated to dryness under a stream of argon. The purity of the product was assessed by analytical HPLC method A.
2.8.5. Cell Binding Assays

MDA-MB-231 (ATCC HTB-26) and MDA-MB-435 (ATCC HTB-129) cells were cultured in Dulbecco’s Modified Eagle’s medium (DMEM) (Invitrogen, 11965) with 10% fetal bovine serum (FBS) (Invitrogen, 12483) and 1% penicillin/streptomycin (P/S) (Invitrogen, 15140). HT-1080 (ATCC CCL-121) cells were cultured in minimum essential medium (MEM) (Invitrogen, 10370) with 10% FBS and 1% P/S, 2 mM L-glutamine (Invitrogen, 25030) and 1 mM sodium pyruvate (Invitrogen, 11360) at 37 °C and 5% CO₂. Coverslips (12 mm round glass coverslips (No.2): VWR, CA89015-724) were sterilized in 70% EtOH for at least 1 h, rinsed with water and aspirated dry. One coverslip was placed in each well in a 24 well plate (VWR, 29444-280). Cells were plated at the density of $1.5 \times 10^5$ cells per well (2 plates) and left to attach and grow for 2 days.

Wells were washed with cold PBS (1 mL) (Invitrogen, AM9624) to remove media and 300 µL of binding buffer (group 1, 2 and 3) [growth media without supplements with 1% BSA (Sigma Aldrich, A7906), 4.5 nM 4b (37 kBq / µL 95 % EtOH)] or binding buffer pre-incubated with 75 nM HMW uPA (EMD chemicals Millipore, 672081, 695 nM) (group 4) was added to each well.

One plate was incubated at 4 °C and one at 37 °C for 2 hours in a humid chamber. Binding buffer was aspirated and 250 µL cold acid wash solution [50 mM Glycine HCl (G2879), 1 × PBS (500 mL), pH 3 with concentrated NaOH (2.788 g in 500 mL)] was added to each well and let sit for 90 sec. Cold neutralization buffer (250 µL) [0.5 M HEPES (H4034), 1 × PBS (500 mL), pH 7.5 with concentrated NaOH (59.575 g
in 500 mL) was added to each well for group 2 and cold PBS (2 × 250 µL) was added to each well for group 1, 3 and 4. Washes/buffers were removed using aspiration and wells were further washed with cold PBS (1 mL).

Each coverslip was transferred to a well in a new 24 well plate (cell side up) and the wells washed with cold PBS (1 mL). 1 % Triton-X in water (350 µL) was added to each well and the plate was incubated at 37 °C for 30 min on a shaker. Cells were mixed by pipetting up and down a few times and cell lysate (300 µL) was transferred to a gamma counting tube and counted for 10 minutes (125I) on a Perkin Elmer Wizard 1470 Automatic Gamma Counter (program 16). Bicinchoninic Acid Protein Assay (BCA) was run for normalization of protein content.

2.8.6. SDS-PAGE

Varying concentrations (0.10 – 0.75 µg) of recombinant uPA (EMD chemicals Millipore, 672081) were incubated on ice for 2 hours with 10× binding buffer (BB) [500 mM Tris (Sigma Aldrich, 154563) pH 7.4 (with HCl), 1.5 M NaCl (Sigma Aldrich, S7653), 5% Pluronic F-68 (Sigma Aldrich, P5556)], water and 6. Following incubation, reaction mixtures were quenched with 6× sample loading dye (SLD) [9% SDS (BioShop, SDS001), 60% glycerol (Caledon Laboatories, 5350-1), 375 mM Tris (Sigma-Aldrich, 154563), 0.015% Bromophenol blue (BioShop, BRO222), 12% 2-mercaptoethanol (BioShop, MER002)] and incubated at room temperature for 30 minutes. After incubation samples were centrifuged for 10 seconds and run on a 10% Mini-PROTEAN TGX Precast gel (Biorad, 4561034) with 10× SDS running buffer [250 mM Tris (Sigma Aldrich, 154563), 1.92 M glycine (BioShop, GLN001), 1% SDS (BioShop, SDS001)].
The gel was run at 80 V until samples were through the stacking gel and then increased up to 120 V until the dye-front was at the bottom of the gel. The gel was left to shrink overnight, dried and visualized on the GE Storm scanner.

2.8.7. Biodistribution Studies

Biodistribution studies for the reversible compounds were carried out on CD1 nude female mice (3-4 weeks old), inoculated with \(2 \times 10^6\) uPAR/uPA expressing either MDA-MB-231 cells or HT-1080 cells in matrigel. Tumour bearing mice were administered 185-370 kBq of 4b, 4e or 4g via tail vein injection. Groups of mice (n=3) were sacrificed at the specified times and tumors, tissues, and fluids collected and the activity counted using a Perkin Elmer Wizard 1470 Automatic Gamma Counter and expressed as percent injected dose per gram (%ID/g).

Biodistribution studies co-administered with uPA were conducted on CD1 nude female mice (4-5 weeks old), which were inoculated with HT-1080 and HT-29 tumours. Four of the six test groups were pre-incubated for thirty minutes at 37 °C with recombinant uPA, prior to injection. Tumour bearing mice were administered 250-290 kBq of 4b via tail vein injection. Groups of mice (n=3) were sacrificed at the specified times and tumors, tissues, and fluids collected and the activity counted using a Perkin Elmer Wizard 1470 Automatic Gamma Counter and expressed as percent injected dose per gram (%ID/g).

Biodistribution studies were carried out on the irreversible inhibitor, 6, in CD1 nude mice expressing HT-1080 human fibrosarcoma tumours. Tumour bearing mice were administered 37-185 kBq of the radiolabeled compound via tail vein injection. Groups of
mice (n=3) were sacrificed at the specified times and tumors, tissues, and fluids collected and the activity counted using a Perkin Elmer Wizard 1470 Automatic Gamma Counter and expressed as percent injected dose per gram (%ID/g).

Biodistribution blocking studies were carried out on 6 in CD1 nude mice expressing HT-1080 human fibrosarcoma tumours. Two groups of mice were utilized – the first were administered 37-185 kBq of the radiolabeled compound alone, whereas the second were administered 37-185 kBq of the radiolabeled compound + 2mg/kg of 2b as in vivo block, via tail vein injection. Following 0.5 h, 2 h, 24 h, 48 h and 96 h uptake periods, groups of mice (n=3 per time point) were sacrificed and the tumours and organs isolated by dissection. Radiochemical uptake in tumour/organ samples was measured and used to generate an in vivo biodistribution pattern, which was expressed as percent injected dose per gram (%ID/g).
CHAPTER THREE: BIOORTHOGONAL CHEMISTRY and PRE-TARGETING STRATEGIES FOR IMAGING UPAR

3.0. Introduction

3.1. Pre-targeting and Bioorthogonal Chemistry

The direct targeting of uPA with inhibitors comes with inherent limitations that are difficult to overcome. uPA is a freely circulating protein and therefore a radiolabeled small molecule may bind to uPA present in the blood where the enzyme-inhibitor complexes never reaches the tumor target. Furthermore, rapid clearance of small molecule inhibitors, coupled with \textit{in vivo} de-iodination, means that these inhibitors may not have long enough circulation time to bind uPA and the tumour.\textsuperscript{62} Given the limited tumor uptake observed for the uPA inhibitors described in chapter 2, despite their high affinity for the protein, an alternative approach for targeting the urokinase system was explored.

Antibodies targeting uPAR have been studied by various research groups and are showing promising results. For example, studies conducted with uPAR antibody 3936 labeled with \textsuperscript{111}In show good tumour uptake, but clearance and target to non-target ratios were not favourable.\textsuperscript{63} One potential alternative, which was explored here, is to use pre-targeting and bioorthogonal chemistry based on catalyst free \textit{in vivo} coupling reactions (Figure 23).\textsuperscript{62,64,65}
Bioorthogonal chemistry involves chemical reactions that are highly selective, biocompatible and are capable of linking two xenobiotics in a living system.\textsuperscript{66} This chemistry has important applications in chemical biology, molecular imaging and medicine.\textsuperscript{62} In particular, tumour pre-targeting takes advantage of the high specificity and selectivity of antibodies, while eliminating the need to use long half-life isotopes associated with the protein’s long circulation times. One particularly successful pre-targeting strategy begins with the injection of an antibody functionalized with a strained alkene such as trans-cyclooctene. The antibody is allowed to accumulate at the tumour and after a given amount of time, an electron-deficient tetrazine tagged with a radioisotope is injected. An inverse-electron-demand Diels-Alder reaction then occurs at sites of high antibody concentrations.\textsuperscript{62}

Kinetics studies have identified the reaction between trans-cyclooctene and tetrazine to have remarkably fast second-order rate constants with values greater than 100 M\textsuperscript{-1} s\textsuperscript{-1}.\textsuperscript{67} This rapid rate is required for the pre-targeting strategy as the reaction speed
must be sufficient to enable capture within the blood stream. The TCO-Tz coupling product is also stable, which is another key element of the system.\textsuperscript{68}

\subsection*{3.2. Radiolabeled Tetrazines}

Antibodies have long been pursued for their potential as molecular imaging agents due to their high specificity and selectivity. However, the long circulation time of antibodies has made it necessary to radiolabel them with isotopes that have longer half-lives, often resulting in higher than optimal background radiation doses to non-target tissues.\textsuperscript{68} Rossin \textit{et al.} demonstrated how pre-targeting combined with bioorthogonal chemical using the Tz-TCO reaction in living animals can circumvent this issue.\textsuperscript{62} They showed it was possible to image colon carcinoma tumours using an $^{111}\text{In}$ labeled tetrazine.

Zeglis \textit{et al.} have reported a complementary system for the construction of radiometalated antibodies based on the bioorthogonal cycloaddition reaction between 3-(4-benzylamino)-1,2,4,5-tetrazine (Tz) and the strained dienophile norbornene. In this system, the antibody of choice is covalently coupled to norbornene and then reacted with tetrazines bearing the chelators 1,4,7,10-tetraazacyclo-dodecane-1,4,7,10-tetraacetic acid (DOTA) or desferrioxamine (DFO) and subsequently radiometalated with $^{64}\text{Cu}$ and $^{89}\text{Zr}$, respectively (Scheme 3).\textsuperscript{69}
Radiolabeled tetrazines derived from a range of SPECT and PET isotopes including $^{11}$C, $^{18}$F, $^{89}$Zr, and $^{111}$In have been reported. Interestingly there have been no reports on tetrazines labeled with $^{99m}$Tc despite its widespread use in diagnostic nuclear medicine although it is noteworthy that a Re-tetrazine complex has recently been reported. Systems based on $^{18}$F, which like $^{99m}$Tc is widely used in nuclear medicine, have been reported but the synthesis of $^{18}$F-tetrazines has proven to be challenging. For instance, due to the harsh reaction conditions typically required for radiolabeling with $^{18}$F, tetrazines, which have been found to be temperature-sensitive, typically degrade.\textsuperscript{70}

As an alternative several groups have reported labeling TCO with $^{18}$F.\textsuperscript{71,72} Wyffels \textit{et al.} evaluated the \textit{in vivo} behavior of TCO radiolabeled with $^{18}$F in an effort to develop a pre-targeting strategy for imaging the brain. Although $^{18}$F-TCO was previously identified as a useful tracer for radiolabeling tetrazine modified targeting molecules, their study refuted this hypothesis. The results of their PET imaging studies showed
homogenous brain uptake with gradually increasing bone uptake, suggesting instability of the compound \textit{in vivo}. Therefore, this construct and approach will have limited utility for pre-targeting strategies.

There remains a need for an approach to preparing $^{99m}$Tc and $^{18}$F tetrazines. Access to suitable derivatives would allow for pre-targeting using two widely accessible isotopes to be used to image antibody distribution. For the purposes of this thesis it could be used with an antibody against uPAR as a new approach to visualizing tumor aggressiveness.

3.3. Hydrazinonicotinic Acid

Although most $^{99m}$Tc radiopharmaceuticals are formed through coordination complexes with simple chelating ligands, there is gaining interest in the ability to alter the ligands surrounding the $^{99m}$Tc core, allowing for tailoring and enhancement of the pharmacokinetic properties of the compound.\cite{74} Hydrazinonicotinic acid (HYNIC) has long been used as a bifunctional agent for radiolabeling biomolecules with $^{99m}$Tc. Abrams \textit{et al.} were the first to describe the synthesis and \textit{in vivo} evaluation of HYNIC bioconjugates in the early 1990s.\cite{75,76} It can be readily added to any biomolecule via a simple active ester coupling and then labeled with $^{99m}$Tc typically through a simple one pot procedure. Since its initial discovery, the utility of HYNIC was investigated extensively for radiolabeling of large biomolecules.\cite{74}

HYNIC can be used to label a range of biomolecules with $^{99m}$Tc through the formation of $^{99m}$Tc-N-N (hydrazino) bonds. The remaining coordination sites around the metal are occupied by a co-ligand such as tricine which is added during labeling (Scheme
4). The nature of the co-ligand can be used to tune the pharmacokinetic profile of the complex – something which is not easily achievable with other $^{99m}\text{Tc}$ ligands. Although many different co-ligands have been tested, the most promising is tricine due to its ability to complex Tc under mild labeling conditions and high polarity which reduces non-specific binding.

![Scheme 4](image)

**Scheme 4** Reaction scheme for radiolabeling of HYNIC peptides with $^{99m}\text{Tc}$ using tricine as coligand. (Reprinted with permission of [77]).

In addition to acting as a ligand that can bind $^{99m}\text{Tc}$, HYNIC can also serve as a prosthetic group for ligating $^{18}\text{F}$ to biomolecules. For instance, Bruus-Jensen *et al.* coupled $^{18}\text{F}$-benzaldehyde to HYNIC-functionalized peptides via formation of a hydrazone linkage with HYNIC. This was accomplished in 85% RCY and the product showed good stability for up to 5 hours in mildly acidic conditions. HYNIC remains one of the few ligands that can be labeled with both $^{18}\text{F}$ and $^{99m}\text{Tc}$ making it an attractive synthon for developing a general purpose radiolabeled tetrazine.
3.4. Objectives

The objective was to develop a method to prepare HYNIC-Tz and to develop a method to prepare $^{99m}$Tc and $^{18}$F labeled tetrazines. Once developed, the stability and biodistribution of the two tetrazines are to be evaluated. This would be followed by evaluating their reactivity towards TCO functionalized biomolecules including a derivative of a monoclonal antibody against human uPAR.

3.5. Chemical Synthesis

A single Tz-HYNIC synthon was synthesized (4, Scheme 5) as the ligand to be used for both routes of radiolabeling. This synthesis was achieved via coupling of the free carboxylic acid of a Boc protected form of HYNIC (1) which was prepared following literature procedure, with the commercially available tetrazine (4-((1,2,4,5-tetrazin-3-yl)phenyl)methanamine (2). Deprotection with TFA resulted in the final product in 65% overall yield, which was then be used for radiolabeling with both $^{99m}$Tc and $^{18}$F.
3.6. Radiolabeling with $^{99m}$Tc

HYNIC-Tz (4) was radiolabeled with $^{99m}$Tc using tricine as a co-ligand (Scheme 6) and the product was ultimately isolated by in 98% purity in 75% radiochemical yield (Figure 24). Optimization involved varying the amount of all reagents as well as the reaction temperature and time. The most significant factor influencing labeling yields was the amount of ligand (Figure 25) where adding more than 100 µg of ligand caused the yield to drop dramatically. The optimal labeling conditions were with 100 µg of compound 4 and allowing the reaction to proceed for 30 minutes at 60 ºC.
Scheme 6 Reaction scheme for the radiolabeling of compound 4 with $^{99m}$Tc.

Figure 24 γ-HPLC chromatogram of 5 following purification by HPLC (HPLC method A).

Figure 25 Radiochemical yield of 5 versus the amount of 4 used during the initial labeling step. Note: The amount of $^{99m}$TcO$_4^-$ was consistent for each labeling experiment (740 MBq).
3.7. Radiolabeling with $^{18}\text{F}$

Radiolabeling of compound 4 with $^{18}\text{F}$ was achieved in a two-step, one pot reaction through the formation of a hydrazone linkage between the free amino group of HYNIC and the free aldehyde of $^{18}\text{F}$-benzaldehyde. 4-Formyl-N,N,N-trimethylanilinium triflate (7, Scheme 7) was synthesized as the aldehyde precursor for the first step of this reaction. Preparation of $^{18}\text{F}$-benzaldehyde followed the method of Bruus-Jensen et al. and was achieved in 65% RCY which is similar to literature yields.78 The reaction did not require isolation of the benzaldehyde, which was used directly in the second step (Scheme 8).
During optimization of the second step, the radiolabeling yield was highly dependent on pH (Figure 26). To maximize the yield, the pH was adjusted to 5 by the dropwise addition of concentrated HCl. When KH$_2$PO$_4$ buffer was used to adjust the pH, the reaction yield dropped significantly, likely due to a competing hydrolysis reaction. Once optimized, the desired $^{18}$F labeled product (9) was obtained in 31% RCY and 99% purity.

![Radiochemical yield with respect to pH of the reaction.](image)

**Figure 26** Radiochemical yield with respect to pH of the reaction.

The non-radioactive reference standard (8, Scheme 9) was synthesized via hydrazone formation between 4-fluorobenzaldehyde and the free hydrazine group of HYNIC. Co-injection of the radiolabeled compound with the non-radioactive reference standard was obtained (Figure 27) to confirm identity of 9.
Scheme 9 Synthesis of non-radioactive reference standard, compound 11.

Figure 27 HPLC analysis of the co-injection of 9 with the reference standard 11. The similar retention time of 15.5 minutes confirms the identity of the radiolabeled compound. The top spectrum is the UV-HPLC chromatogram and the bottom spectrum is the gamma chromatogram (λ = 254nm). Note that the detectors are connected in series (HPLC method B).

3.8. Stability Studies

In order to test the stability of 5 and 9, the radiolabeled products were dissolved in saline and analyzed by analytical HPLC as a function of time. Stability studies were performed following isolation by semi-preparative HPLC, where the pure products were dried using a Biotage evaporation system and reconstituted in 0.9% saline (5.55 MBq /
mL saline for 5 and 11.1 MBq / mL saline for 9). Stability studies were carried out for six hours for compound 5 (Figure 28) and two hours for compound 9 (Figure 29).

Figure 28 γ-HPLC chromatograms of 5 in saline at 0.5 h, 1 h, 4 h and 6 h (top to bottom, HPLC method B).
Since no non-radioactive reference standard can be synthesized for the $^{99m}$Tc-labeled compound, it was important to find a way to test that the radiolabeled complex was actually the desired product. To this end, compound 5 was incubated at room temperature with 120 ng TCO and the reaction mixture assessed by analytical HPLC after 15 min. Results showed formation of a new broad peak, indicating that the compound did indeed undergo the ligation reaction between the TCO and tetrazine (Figure 30). The

Figure 29 $\gamma$-HPLC chromatograms of 11 in saline at 1 h, 1.5 h and 2 h (top to bottom, HPLC method B).

3.9. Reaction with (E)-Cyclooct-4-enol (TCO)

Since no non-radioactive reference standard can be synthesized for the $^{99m}$Tc-labeled compound, it was important to find a way to test that the radiolabeled complex was actually the desired product. To this end, compound 5 was incubated at room temperature with 120 ng TCO and the reaction mixture assessed by analytical HPLC after 15 min. Results showed formation of a new broad peak, indicating that the compound did indeed undergo the ligation reaction between the TCO and tetrazine (Figure 30). The
same test was also completed for the $^{18}$F derivative (9), confirming its reaction with TCO (Figure 31).

**Figure 30** γ-HPLC chromatograms of the reaction between 5 and TCO (top) and 5 alone (bottom).
Figure 31 γ-HPLC chromatograms of the reaction between 9 and TCO (top) and 9 alone (bottom).

3.10. TCO Anti-uPAR Antibody Conjugation

The TCO-conjugated antibody (TCO–anti-uPAR) was prepared by combining an excess of TCO–NHS with a murine monoclonal antibody for uPAR (Scheme 10). Success was confirmed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOFMS), which showed an average of 3.4 TCO groups per antibody in the product (Figure 32).
Scheme 10 Schematic for the conjugation of anti-uPAR antibody with \((E)\)-cyclooct-4-enyl 2,5-dioxo-1-pyrrolidinyl carbonate.

Figure 32 MALDI-TOF analysis. The top spectrum is for the uPAR antibody alone. Second spectrum is for the antibody +10 equivalents of \((E)\)-cyclooct-4-enyl 2,5-dioxo-1-pyrrolidinyl carbonate. The third spectrum is for the uPAR antibody +20 equivalents of \((E)\)-cyclooct-4-enyl 2,5-dioxo-1-pyrrolidinyl carbonate. Both the second and third spectrum show an average of 3.4 transcyclooctene conjugated per antibody.
3.11. Radio-TLC

Since no non-radioactive reference standard is able to be synthesized for compound 5, radio-TLC was performed with TCO-conjugated anti-uPAR antibody. Rapid conjugation successfully showed that the radiolabeled compound did bind to the antibody, confirming the identity of 5 (Figure 33 and 34).

![Radio-TLC of compound 5](image)

**Figure 33** Radio-TLC of compound 5. Note: Baseline of 50 mm used.
Figure 34 Radio-TLC of 5, conjugated with TCO-anti-uPAR antibody, as demonstrated by the new peak formed at 50 mm. Note: baseline of 50 mm used.

3.12. Biodistribution Studies

Biodistribution studies were performed for both the $^{99m}$Tc (5) and $^{18}$F (9) derivatives in healthy mice to assess clearance times and pathway. Results for 5 showed clearance via the gall bladder and intestines with slow clearance from the blood (4.9 %ID/g at 0.5 h, 4.3 %ID/g at 1 h, 3.8 %ID/g at 2 h and 1.7 %ID/g at 6 h) (Figure 35). Results for 9 showed rapid clearance from the blood (<1% at 1 h) where the majority of activity was found in the bladder and urine (Figure 36). At 2 hrs, the biodistribution of the $^{18}$F-labeled tetrazine was similar to others reported in the literature with dominant uptake in the urine/bladder and similar distribution found in most organs, tissues and
fluids with the exception of the gall bladder. For the $^{99m}$Tc complex, the distribution showed longer blood circulation and higher liver and intestinal uptake than for 9. These results are consistent with the distribution of other HYNIC-tricine derivatives including peptide conjugates.\textsuperscript{79–82} The $^{99m}$Tc compound has, as mentioned, the advantage that the nature of the coligand can be adjusted to vary the distribution.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{\textit{In vivo} biodistribution of 5 in female CD1 mice at 0.5 h, 1 h, 2 h and 6 h PI. Data expressed as %ID/g.}
\end{figure}
3.13. Summary and Future Work

In conclusion, a single tetrazine synthon has been developed for use as the primary precursor for both $^{99m}$Tc and $^{18}$F labeling. This is the first tetrazine of its kind and will allow for SPECT and PET imaging of TCO-functionalized antibodies. Optimization of radiolabeling procedures has been performed and *in vitro* stability studies in saline conducted. Successful ligation with TCO has been demonstrated as well as biodistribution studies in healthy mice performed. Both the $^{99m}$Tc and $^{18}$F derivatives show promise for use in pre-targeting strategies for the development of a radiopharmaceutical targeting the uPA system using TCO-modified anti-uPAR antibodies.
Future work should include assessing stability in plasma for both the $^{99m}$Tc and $^{18}$F derivatives and measuring the kinetics studies of both the non-radioactive and radioactive derivatives to ensure adequate rate constants for \textit{in vivo} coupling. In parallel \textit{in vitro} assay for both derivatives should also be conducted to assess their ability to react with TCO-tagged cells. For the system of interest here, the studies should be performed using A431 cells (uPAR positive) and MCF-7 cells (uPAR negative) treated with a TCO-conjugated anti-uPAR antibody. With success this would be followed by biodistribution studies in tumour bearing mouse models.

3.14. Experimental

3.14.1. General

Unless otherwise stated, all chemical reagents were purchased and used as received from Sigma-Aldrich without further purification. Solvents were purchased from Caledon. $^1$H and $^{13}$C NMR spectra were recorded on a Bruker AV 600 or AV 200 spectrometer. $^1$H chemical shifts are reported in ppm relative to the residual proton signal of the NMR solvents. Coupling constants ($J$) are reported in Hertz (Hz). $^{13}$C chemical shifts are reported in ppm relative to the carbon signal of the NMR solvents. High resolution mass spectra (HRMS) were obtained on a Waters QToF Ultima Global spectrometer. $^{99m}$Pertechnetate [$^{99m}$TcO$_4$]$^-$ was obtained in saline from a $^{99}$Mo/$^{99m}$Tc generator supplied by Lathenus Medical Imaging. [$^{18}$F]fluoride Kryptofix 2.2.2. complex was provided by the CPDC cyclotron at McMaster University.

Reverse phase analytical HPLC was performed using a Waters 2489 HPLC equipped with a Waters 2489 UV/Vis ($\lambda = 254$ nm or $350$ nm) and a Bioscan Glow count
gamma detector (model 106). Spectra were recorded and processed on Empower 2 software (Waters). Semi-preparative HPLC method A was performed using a C18 reverse phase Phenomenex Gemini column (250 × 10.00 mm, 5 micron) with HPLC grade water (solvent A) and acetonitrile (solvent B) used as eluents. This method had a flow rate of 4 ml/min and a 95 to 5% gradient (Solvent A : Solvent B) over 30 minutes. Analytical HPLC method B was performed using a C18 reverse phase Phenomenex column (25 × 4.60 mm, 5 micron) with HPLC grade water containing 0.1% TFA (Solvent A) and acetonitrile containing 0.1% TFA (Solvent B) used as eluents. This method had a flow rate of 1 mL/min and a 95 to 5% gradient (Solvent A: Solvent B) over 30 minutes.

3.14.2. Synthetic Procedures

Synthesis of 3:

\[
\begin{align*}
\text{O} & \quad \text{O} \\
\text{N} & \quad \text{N} \\
\text{N} & \quad \text{N} \\
\text{N} & \quad \text{N} \\
\text{H} & \quad \text{H} \\
\text{O} & \quad \text{O} \\
\end{align*}
\]

The synthesis was adapted from a procedure reported by Zeglis and coworkers.\(^6\) 3-(4-Benzy lamino)-1,2,4,5-tetrazine (13 mg, 0.06 mmol) was dissolved in dimethylformamide (DMF, 2 mL), and diisopropylethylamine (DIPEA, 21 μL, 0.12 mmol) added. After 15 min of stirring at RT, the pink DMF solution was added to a second, premixed solution of 6-BOC-hydrazinopyridine-3-carboxylic acid (30 mg, 0.12 mmol) and benzotriaz- zole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluoro-phosphate (PYBOP, 84 mg, 0.16 mmol) in DMF (2 mL). The combined solutions were stirred overnight under argon. The
reaction mixture was concentrated to dryness under reduced pressure, giving pink solid, which was dissolved in a minimum amount of ethyl acetate and the desired product isolated as a pink solid by silica gel chromatography using ethyl acetate / hexane (80:20) as the eluent (16 mg, 65%).

$^1$H NMR (600 MHz, CD$_3$OD): 1.49 (s, 9 H), 4.69 (s, 2 H), 6.73 (d, J = 8.8 Hz, 1 H), 7.62 (d, J = 8.4 Hz, 2 H), 8.06 (dd, J = 8.8 Hz, J = 2.3 Hz, 1 H), 8.57 (d, J = 8.4 Hz, 2 H), 8.62 (d, J = 1.9 Hz, 1 H), 10.31 (s, 1 H). $^{13}$C NMR (600 MHz, CD$_3$OD): 29.45, 44.98, 82.74, 107.75, 123.03, 130.21, 133.14, 139.24, 146.67, 149.80, 159.26, 160.10, 164.25, 168.50 and 169.27. HRMS (ESI$^+$): for C$_{20}$H$_{23}$N$_8$O$_3$ calculated: 423.1815 found: 423.1886. HPLC $R_t$ = 10 min.
Figure 37 $^1$H NMR for compound 3.
Figure 38 $^{13}$C NMR for compound 3.
Figure 39 HRMS for compound 3.

Deprotection of 3 to give 4:

\[
\begin{align*}
\text{H}_2\text{N} &- \text{N} - \text{H} \\
\text{O} &- \text{N} - \text{H} \\
\text{H}_2\text{N} &- \text{N} - \text{N} - \text{N} - \text{N}
\end{align*}
\]

The deprotection was adapted from a procedure by Yang and coworkers.\(^8^3\) Tz-Hynic-Boc (3) (15 mg, 0.036 mmol) was dissolved in 50% v/v TFA/DCM while in an ice bath and the mixture stirred over ice for one hour and then at room temperature for another hour. The reaction mixture was then concentrated to dryness yielding a pink solid (10 mg, 86%).\(^1\)H NMR (200 MHz, CD\(_3\)OD): 4.70 (s, 2 H), 6.99 (d, J = 9.5 Hz, 1 H), 7.62 (d, J =
8.4 Hz, 2 H), 8.25 (dd, J = 9.3 Hz, J = 2.1 Hz, 1 H), 8.51 (d, J = 1.6 Hz, 1 H), 8.57 (d, J = 8.3 Hz, 2 H), 10.33 (s, 1 H). HPLC R<sub>t</sub> = 4 min. MS (ESI<sup>+</sup>) for C<sub>15</sub>H<sub>14</sub>N<sub>8</sub>O calculated: 323.13 found: 323.1.

Figure 40 <sup>1</sup>H NMR for compound 4.

Figure 41 MS for compound 4.
Synthesis of 4-formyl-N,N,N-trimethylanilinium triflate (7):

The synthesis was adapted from a procedure by Bruus-Jensen and coworkers.\textsuperscript{78} 4-(Dimethylamino) benzaldehyde (0.5 g, 3.4 mmol) was dissolved in 3 mL DCM and the solution cooled in an ice bath for 5 minutes under argon. Methyl trifluoromethane sulfonate (0.4 mL, 0.7 mmol) was added dropwise (reaction turns yellow) and the mixture stirred at room temperature for 3 hours. The reaction mixture was triturated with hot DCM and the product collected by filtration as a white powder (40 mg, 35 % yield). Compound 5 showed m.p. 107 – 110 °C; \textsuperscript{1}H NMR (200 MHz, MeOD): 3.63 (s, 9 H), 8.12 – 8.22 (m, 4 H), 10.11 (s, 1 H).
Figure 42 $^1$H NMR for compound 7.

**Synthesis of 11:**

The synthesis was adapted from a procedure by Bruus-Jensen and coworkers. $^{78}$ HYNIC-Tz (4) (13.6 mg, 0.042 mmol) was dissolved in 500 µL H$_2$O and 500 µL DMF. 4-fluorobenzaldehyde (7) (6.77 µL, 0.063 mmol) was added and the reaction was stirred at 70 ºC for one hour. The reaction was allowed to cool to room temperature before being
extracted with $4 \times 10$ mL H$_2$O. The organic phase was dried over anhydrous sodium sulfate and the solvent was removed by rotary evaporation to afford a red solid. The product was dissolved in a minimal amount of ethyl acetate and purified on a silica column (80% ethyl acetate / hexanes). HRMS (ESI$^+$): for C$_{22}$H$_{17}$FN$_8$O calculated: 429.1576 found: 429.1570.

**Figure 43** HRMS spectra for compound 11.

### 3.14.3. General Radiolabeling Procedure for $^{99m}$Tc

HYNIC-Tz (4) (100 µg, 310 nmol) was dissolved in water (100 µL) in an Eppendorf tube. A tricine solution (500 µL, 100 mg / mL in water), 500 µL $^{99m}$TcO$_4^-$ (20 mCi, 740 MBq) and 10 µL of a stannous chloride dihydrate solution (3 mg / mL ethanol) were added. The solution was vortexed for 2 minutes and heated at 60 ºC for 0.5 hours, at which time the reaction was allowed to cool to room temperature and the desired product (5) isolated by semi-preparative HPLC (method A).
3.14.4. General Radiolabeling Procedure for $^{18}$F

A solution of $[^{18}\text{F}]$fluoride Kryptofix 2.2.2. complex (1740 MBq) was transferred to a 5 mL pyrex® reaction vial and the solution evaporated to dryness by heating at 90 °C in an oil bath under a stream of nitrogen gas. Azeotropic drying was performed by the addition of six 1 mL portions of acetonitrile over a 20 minute period until a brown film was seen on the bottom of the reaction vessel. After cooling to room temperature, 7 (2 mg, 6.4 µmol) dissolved in 100 µL of anhydrous DMSO was transferred to the reaction vial. The vial was capped and heated to 90 °C in an oil bath for 5 minutes with stirring and then allowed to cool to room temperature. Hynic-Tz (4) (2 mg, 6.2 µmol) was dissolved in 200 µL anhydrous DMSO and added to the reaction vessel. Concentrated hydrochloric acid (approximately one drop) was used to adjust the pH to 5.5. The solution was heated to 70 °C in an oil bath for 10 minutes and the desired product isolated by semi-preparative HPLC (method A).

3.14.5. TCO Anti-uPAR Antibody Conjugation

The TCO-conjugated antibody (TCO–anti-uPAR) was prepared by combining an excess (20 equivalents) of commercially available (E)-cyclooct-4-enyl-2,5-dioxopyrrolidin-1-yl carbonate (TCO–NHS, 1.78 µg, 6.67 nmol) with a murine monoclonal antibody, subclass IgG2a recognizing the human urokinase plasminogen activator receptor (uPAR) (American Diagnostica Inc., Product No. 3936) at 4 °C overnight. The reaction was conducted in PBS where the pH was adjusted to 8.5–9.0 using 1 M Na$_2$CO$_3$. The desired product was isolated using a 30 kDa centrifugal filter (Amicon Ultra-0.5) and washed with PBS three times. The weight of the sample before
and after conjugation was determined by MALDI-TOFMS (MALDI Bruker UltraflexXtreme Spectrometer) indicating an average of 3.4 TCO groups per antibody.

3.14.6. Radio-TLC

TCO-conjugated anti-uPAR antibody (10 µg in 20 µL PBS) was incubated with compound 5 (1.4 MBq in 20 µL PBS) for 15 minutes. In parallel, compound 5 (1.4 MBq in 20 µL PBS) was incubated with 20 µL PBS for 15 minutes. Both samples were run on iTLC-SG glass microfiber chromatography paper (Agilent Technologies, SGI0001) plates for 3 minutes using citrate buffer (0.1 M, pH 5.5). Plates were analyzed on a Bioscan AR-2000 Imaging Scanner.

3.14.7. Biodistribution Studies

Biodistribution studies of 5 were performed using 4-5 week old female CD1 mice (Charles River Laboratories, QC, Canada). Mice were administered approximately 0.54 MBq of 5 (100 µL in 0.9 % NaCl) via tail vein injection and groups of mice were sacrificed at t = 0.5 h, 1 h, 2 h and 6 h (n = 3 per time point) and tissues and fluids collected and the activity counted using a Perkin Elmer Wizard 1470 Automatic Gamma Counter and expressed as percent injected dose per gram (%ID/g).

Biodistribution studies of 9 was performed using 7-8 week old female CD1 mice (Charles River Laboratories, QC, Canada). Mice were administered approximately 0.88 MBq of 9 (100 µL in 0.9% NaCl) via tail vein injection and groups of mice were sacrificed at t = 0.5 h, 1 h, 2 h and 4 h (n = 3 per time point) and tissues and fluids collected and the activity counted using a Perkin Elmer Wizard 1470 Automatic Gamma Counter and expressed as percent injected dose per gram (%ID/g).
REFERENCES


(59) Zeslawska, E.; Jacob, U.; Schweinitz, A.; Coombs, G.; Bode, W.; Madison, E. J. 

(60) Hang, N.; Ranson, M.; Saunders, D. N.; Liang, X.; Bunn, C. L.; Baker, M. S. 


(62) Rossin, R.; Renart Verkerk, P.; van den Bosch, S. M.; Vuldres, R. C. M.; Verel, I.; 


(64) Sillaber, C.; Baghestanian, M.; Hofbauer, R.; Virgolini, I.; Bankl, H. C.; Füreder, 
W.; Agis, H.; Willheim, M.; Leimer, M.; Scheiner, O.; Binder, B. R.; Kiener, H. 

Metastasis 2006, 21 (1), 20–27.


53 (23), 5805–5809.

(68) Rossin, R.; van den Bosch, S. M.; Ten Hoeve, W.; Carvelli, M.; Versteegen, R. 

(69) Zeglis, B. M.; Mohindra, P.; Weissmann, G. I.; Divilov, V.; Hilderbrand, S. A.; 


(71) Liu, S.; Hassink, M.; Selvaraj, R.; Yap, L.-P.; Park, R.; Wang, H.; Chen, X.; Fox, 

(72) Li, Z.; Cai, H.; Hassink, M.; Blackman, M. L.; Brown, R. C. D.; Conti, P. S.; Fox, 


