Panitumumab modified with metal chelating polymers (MCPs) complexing indium-111 and lutetium-177 as theranostics for pancreatic cancer

By

Sadaf Aghevlian

A thesis is submitted in conformity with the requirements for the degree of Doctor of Philosophy

Graduate Department of Pharmaceutical Sciences
University of Toronto

© Copyright by Sadaf Aghevlian, 2019
Panitumumab modified with metal chelating polymers (MCPs) complexing indium-111 and lutetium-177 as theranostics for pancreatic cancer

Sadaf Aghevlian
Doctor of Philosophy
Graduate Department of Pharmaceutical Sciences
University of Toronto
2019

Abstract

The epidermal growth factor receptor (EGFR) is overexpressed in more than 90% of pancreatic cancer (PnCa) patients. A metal-chelating polymer (MCP) with on average 13 DOTA (tetraazacyclododecane-1,4,7,10-tetraacetic acid) chelators for complexing the β-particle emitter, $^{177}$Lu and Auger electron-emitter, $^{111}$In and 10 polyethylene glycol (PEG) chains was conjugated to monoclonal antibody, panitumumab to target EGFR on PANC-1 human PnCa cells. Linking panitumumab to MCPs enabled dual labeling with $^{111}$In and $^{177}$Lu at a higher SA (55.9± 4.8 MBq/μg and 20.5± 4.2 MBq/μg) compared to panitumumab-DOTA (4.4± 0.3 MBq/μg and 2.2± 0.3 MBq/μg), with preserved EGFR binding (2.2 ± 0.6 nmol/L and 1.0 ± 0.4 nmol/L; respectively) and comparable tumour (6.9 ± 1.3% ID/g and 6.6 ± 3.3%ID/g) and normal tissue localization except for the liver uptake which was 3-fold higher for panitumumab-MCP at 72 h post injection (p.i.) in NOD/SCID mice with s.c. PANC-1 xenografts as confirmed by microSPECT/CT imaging. $^{177}$Lu-labeled radioimmunoconjugates (RICs) were more effective for killing PANC-1 cells in vitro than $^{111}$In-RICs with no significant differences between panitumumab-DOTA and panitumumab-MCP. For therapy, NOD/SCID mice or NRG mice with s.c. PANC-1 tumors were administered three amounts (10 MBq; 10 μg) of panitumumab-MCP-$^{111}$In or panitumumab-DOTA-$^{111}$In separated by 3 weeks or a single amount (6 MBq; 10 μg) of panitumumab-MCP-$^{177}$Lu or panitumumab-DOTA-$^{177}$Lu, respectively. Tumour growth was assessed by a tumour growth index (TGI). Panitumumab-MCP-$^{111}$In or panitumumab-DOTA-$^{111}$In inhibited tumour growth in NOD/SCID mice (TGI at 43 days = 3.9 ± 0.3 and 3.0 ± 0.4, respectively; P> 0.05) compared to normal saline and panitumumab treated mice (TGI = 9.8 ± 1.6, 9.9 ± 1.4, respectively; P>0.5). Similarly, panitumumab-MCP-$^{177}$Lu and panitumumab-
DOTA-$^{177}$Lu inhibited tumour growth in NRG mice (TGI at 33 days = 2.5 ± 0.3 and 1.8± 0.3; respectively; P> 0.05) compared to normal saline and panitumumab treated mice (5.8 ± 0.9 vs. 6.1 ± 2.7, respectively; P>0.05). $^{177}$Lu-RICs deposited more absorbed doses in tumours (2-4 fold) and normal organs compared to $^{111}$In-RICs. Low absorbed doses in the normal organs for $^{111}$In allow for dose escalation. Panitumumab conjugates labeled with $^{177}$Lu or $^{111}$In are promising RIT agents for treatment of EGFR positive cancers.
Acknowledgments

First and foremost I’d like to convey my utmost gratitude to my supervisor Dr. Raymond Reilly for the lifetime opportunity to come to Canada and peruse my doctoral studies under his supervision. I am sincerely thankful for not only his scientific contributions to develop and complete my research project, but also for being such a wonderful person and great role model.

I would also like to thank the members of my advisory committee, Dr. Mitchell Winnik, Dr. David Hedley, and Dr. Stephan Angers for their helpful suggestions, comments and encouragements. Thank you for challenging me to think critically about my research.

I would like to thank my past and present colleagues, especially Dr. Conrad Chan, Dr. Zhongli Cai, Dr. Yijie Lu, Ms. Deborah Scollard and Ms. Teesha Kemal for sharing their knowledge and friendship.

I wish to extend my deepest gratitude to my family, my brother Sohrab, my parents Reza and Maryam and my dear uncle Khalil for their unwavering love and support throughout my whole life. I am forever blessed to have you in my life. And a special thank you to my husband Mehrdad for his love, patience and care. Thank you for believing in me and supporting all of my hopes and dreams.
Table of contents

Abstract ................................................................................................................................. ii
Acknowledgments ............................................................................................................... iv
Table of contents .............................................................................................................. v
List of tables ..................................................................................................................... ix
List of figures .................................................................................................................... xi
List of abbreviations ......................................................................................................... xiv
Chapter 1: Introduction ..................................................................................................... 1
  1. Introduction .................................................................................................................. 2
    1.1 Current status in the detection, diagnosis and treatment of pancreatic cancer .......... 2
      1.1.1 Pancreatic cancer epidemiology and etiology .................................................... 2
      1.1.2 Detection, diagnosis and staging of PnCa ......................................................... 2
    1.2 PnCa biology ........................................................................................................... 5
      1.2.1 The genomic landscape of PnCa ...................................................................... 5
      1.2.2 PnCa stem cells ............................................................................................. 7
      1.2.3 The tumour microenvironment ...................................................................... 7
      1.2.4 Circulating tumour cells .............................................................................. 9
    1.3 Treatment of pancreatic cancer ........................................................................... 9
      1.3.1 Surgery ........................................................................................................... 9
      1.3.2 Chemotherapy ............................................................................................ 10
    1.4 Diagnostic imaging modalities for PnCa ........................................................... 19
      1.4.1 Contrast-enhanced abdominal ultrasound ....................................................... 19
      1.4.2 MDCT ......................................................................................................... 19
      1.4.3 Endoscopic Ultrasound Guided Fine Needle Aspiration (EUS/EUS-FNA) ........ 20
      1.4.4 MRI .......................................................................................................... 20
      1.4.5 Nuclear medicine imaging ........................................................................... 21
      1.4.6 Summary of clinical roles for imaging in PnCa ............................................. 32
    1.5 Therapeutic targets for PnCa ............................................................................. 34
    1.6 Radioimmunotherapy of PnCa by targeting EGFR ............................................ 40
      1.6.1 Types of radiation used in radioimmunotherapy .............................................. 41
      1.6.2 Clinical trials with RICs ............................................................................. 55
    1.7 Increasing the specific activity (SA) ................................................................. 58
      1.7.1 Dendrimer ................................................................................................... 59
      1.7.2 Metal Chelating Polymer (MCP) .................................................................. 62
Chapter 2: Panitumumab Modified with Metal-Chelating Polymers (MCP) Complexed to $^{111}$In and $^{177}$Lu – An EGFR-Targeted Theranostic for Pancreatic Cancer ......................................................... 66

2. Abstract .......................................................................................................................... 68

2.1 Introduction .................................................................................................................. 71

2.2 Materials and Methods .............................................................................................. 73

2.2.1 Cell Culture and Tumour Xenografts .................................................................... 73

2.2.2 Radioimmunoconjugates (RICs) .......................................................................... 74

2.2.3 Measurement of Hydrodynamic Radius ................................................................ 76

2.2.4 EGFR Immunoreactivity ....................................................................................... 77

2.2.5 Stability of the RICs .............................................................................................. 78

2.2.6 Imaging and Biodistribution Studies .................................................................... 79

2.3 Statistical Analysis ..................................................................................................... 79

2.4 Results ......................................................................................................................... 80

2.4.1 Radioimmunoconjugates ....................................................................................... 80

2.4.2 Hydrodynamic Radius ......................................................................................... 84

2.4.3 EGFR Immunoreactivity ....................................................................................... 87

2.4.4 Radioimmunoconjugate Stability ......................................................................... 89

2.4.5 Biodistribution and Imaging Studies ................................................................... 93

2.5 Discussion ................................................................................................................... 97

2.6 Conclusions ................................................................................................................ 101

Chapter 3: Radioimmunotherapy of PANC-1 Human Pancreatic Cancer Xenografts in NRG Mice with Panitumumab Modified with Metal-Chelating Polymers (MCP) Complexed to $^{177}$Lu ........................................................... 102

3. Abstract ......................................................................................................................... 104

3.1 Introduction ................................................................................................................ 106

3.2 Materials and Methods ............................................................................................ 108

3.2.1 Cell culture and tumour xenografts .................................................................... 108

3.2.2 Radioimmunoconjugates ....................................................................................... 108

3.2.3 In vitro cytotoxicity studies ................................................................................. 108

3.2.4 Subcellular distribution and microdosimetry ....................................................... 109

3.2.5 Evaluation of normal tissue toxicity .................................................................... 110

3.2.6 Radioimmunotherapy studies .............................................................................. 111

3.2.7 Tumour and normal organ dosimetry ................................................................. 111

3.3 Statistical analysis ..................................................................................................... 112
Chapter 5: Summary and Future Directions

4. Pancreatic Cancer Xenografts in NOD/SCID and NRG Mice Complexed to Panitumumab for Radioimmunotherapy of EGFR-Positive PANC-1 Human Pancreatic Cancer Xenografts in NOD/SCID and NRG Mice

Chapter 4: Comparison of Auger Electron-Emitting $^{111}$In- or $\beta$-Particle-Emitting $^{177}$Lu Complexed to Panitumumab for Radioimmunotherapy of EGFR-Positive PANC-1 Human Pancreatic Cancer Xenografts in NOD/SCID and NRG Mice

4. Abstract ............................................................................................................................... 134

4.1 Introduction ....................................................................................................................... 136

4.2 Materials and methods .................................................................................................... 139

4.2.1 Cell Culture and tumour xenografts ........................................................................... 139

4.2.2 Radioimmunoconjugates (RICs) .............................................................................. 140

4.2.3 Clonogenic Survival (CS) ....................................................................................... 140

4.2.4 Assessment of DNA damage .................................................................................... 141

4.2.5 Subcellular distribution and Cellular Dosimetry ....................................................... 141

4.2.6 Evaluation of Normal Tissue Toxicity ...................................................................... 142

4.2.7 Radioimmunotherapy (RIT) Studies ........................................................................ 143

4.2.8 Tumour and normal organ dosimetry ...................................................................... 143

4.3 Statistical Analysis .......................................................................................................... 144

4.4 Results ............................................................................................................................... 144

4.4.1 Clonogenic Survival (CS) ....................................................................................... 144

4.4.2 Assessment of DNA damage .................................................................................... 146

4.4.3 Subcellular distribution and cellular dosimetry ....................................................... 148

4.4.4 Evaluation of Normal Tissue Toxicity ...................................................................... 153

4.4.5 Radioimmunotherapy (RIT) studies ........................................................................ 155

4.4.6 Tumour and Normal Organ Dosimetry .................................................................... 157

4.5 Discussion ......................................................................................................................... 160

4.6 Conclusion ....................................................................................................................... 164

4.7 Acknowledgments ............................................................................................................ 164

Chapter 5: Summary and Future Directions ......................................................................... 165
5 Thesis Conclusion and Summary of Findings ................................................................. 166
  5.1.1 Chapter 2 ........................................................................................................... 167
  5.1.2 Chapter 3 ........................................................................................................... 168
  5.1.3 Chapter 4 ........................................................................................................... 169
  5.1.4 Future directions ............................................................................................. 171
6 Appendices ................................................................................................................ 174
  6.1 Appendix A: Therapeutic efficacy of the $^{111}$In- and $^{177}$Lu-labeled RICs side by side .... 175
    6.1.1 Appendix A1. Clonogenic survival .................................................................... 176
    6.1.2 Appendix A2: DNA damage assessment using $\gamma$-H2AX assay ...................... 177
    6.1.3 Appendix A3: Absorbed dose in the nucleus of PANC-1 cells for $^{111}$In- or $^{177}$Lu-
        RICs .................................................................................................................... 178
    6.1.4 Appendix A4: Toxicity study ............................................................................ 179
    6.1.5 Appendix A5: RIT study with $^{177}$Lu-RICs ....................................................... 180
    6.1.6 Appendix A6: RIT study with $^{111}$In-RICs ....................................................... 181
    6.1.7 Appendix A7: Tumour and normal organs dosimetry ...................................... 182
  6.2 Appendix B. Immunohistochemical staining of PANC-1 xenografts obtained from
      NOD/SCID mice and NRG mice for characterization of the tumour microenvironment...... 183
References .................................................................................................................. 185
List of tables

Table 1-1. TNM staging system for PnCa. ................................................................. 4
Table 1-2. Physical properties of some positron emitting radionuclides used in PET imaging. . 25
Table 1-3. Physical properties of some of radionuclide used in SPECT imaging. .................. 30
Table 1-4. Potential molecular targets with frequency of genetic aberration on PnCa and their targeted agents. ....................................................................................... 37
Table 1-5. Phase II-III clinical trials with targeted therapies in PnCa. ................................ 39
Table 1-6. α-particle emitters for conjugation to mAbs for RIT of cancer............................ 42
Table 1-7. Auger electron emitters for conjugation to mAb for RIT of cancer...................... 46
Table 1-8. β-particle emitters for conjugation to mAb for RIT of cancer. ............................ 48
Table 1-9. Ongoing clinical trials for cancer treatment using ⁷⁷Lu labeled RICs and radiopeptides. .................................................................................................................. 50
Table 2-1. Labelling Efficiency and Specific Activity of Panitumumab-MCP and Panitumumab-DOTA with ¹¹¹In or ¹⁷⁷Lu. ................................................................. 83
Table 2-2. Hydrodynamic radii and retention times for PEG and protein standards by size exclusion chromatography ................................................................. 86
Table 3-1. Absorbed doses in the nucleus of PANC-1 cells by panitumumab-MCP-177Lu ........ 118
Table 3-2. Cumulative radioactivity in source organs for panitumumab-MCP-177Lu injected i.v. in NRG mice with s.c. PANC-1 tumour xenografts. ............................................. 126
Table 3-3. Estimated radiation absorbed doses in the tumour and normal organs in NRG mice with s.c. PANC-1 xenografts injected with panitumumab-MCP-177Lu ......................... 127
Table 4-1. Cumulative radioactivity in the source component after incubation of PANC-1 cells with 1.2 MBq of ¹¹¹In-labeled RICs in the absence or presence of 50-fold excess unlabeled panitumumab for 24 h at 37 °C ................................................................. 151
Table 4-2. Cumulative radioactivity in the source component after incubation of PANC-1 cells with 1.2 MBq of panitumumab-DOTA-177Lu in the absence or presence of 50-fold excess unlabeled panitumumab for 24 h at 37 °C ................................................................. 152
Table 4-3. Estimated absorbed doses in the tumour and normal organs in mice with s.c. PANC-1 xenografts injected with ¹¹¹In- or ¹⁷⁷Lu-RICs. ................................................................. 159
Table S 1. Absorbed dose in the nucleus following incubation of cells with 1.2 MBq of ¹¹¹In-RICs in the absence or presence of excess of unlabeled panitumumab for 24 h at 37°C.......178
Table S 2. Absorbed dose in the nucleus following incubation of cells with 1.2 MBq of $^{177}$Lu-RICs in the absence or presence of excess of unlabeled panitumumab for 24 h at 37°C ........178

Table S 3. Estimated absorbed doses in the tumour and normal organs in mice with s.c. PANC-1 xenografts injected with $^{111}$In- or $^{177}$Lu-RICs based on biodistribution study at different time points.................................................................159

Table S 4. Staining of various antigens in PANC-1 tumour sections.................................183
List of figures

Figure 1-1. Signaling pathways with mutation in PnCa. ................................................................. 6
Figure 1-2. Methylation of deoxyuridine monophosphate to deoxythymidine monophosphate by thymidylate synthase.......................................................... 11
Figure 1-3. The chemical structure of gemcitabine. ................................................................. 13
Figure 1-4. Schematic structure of Nab-paclitaxel or Abraxane. ........................................... 16
Figure 1-5. Schematic structure of Irinotecan liposome injection (ONIVYDE). ...................... 18
Figure 1-6. PET imaging system and types of coincidences in PET. ...................................... 23
Figure 1-7. Composition of the SPECT camera. ..................................................................... 28
Figure 1-8. Potential targets in pancreatic tumourigenesis..................................................... 35
Figure 1-9. The chemical structure of $^{177}$Lu-DOTATATE .................................................. 52
Figure 1-10. Types of radiation used in RIT ........................................................................ 54
Figure 1-11. The protocol schema of RIT with $^{90}$Y-hPAM4 ................................................ 56
Figure 1-12. Three main parts of a dendrimer ................................................................... 59
Figure 1-13. The structure of a dendrimer with multiple surface functional groups ........... 60
Figure 1-14. The structure of a metal chelating polymer...................................................... 63
Figure 2-1. Chemical structure of the hydrazino nicotinamide metal chelating polymer (HyNic-MCP). .......................................................................................... 75
Figure 2-2. Synthesis of panitumumab-MCP conjugates. ..................................................... 81
Figure 2-3. Determination of hydrodynamic radius of panitumumab-DOTA, panitumumab-MCP and unconjugated MCP ........................................................................... 85
Figure 2-4. Binding of panitumumab-DOTA-$^{177}$Lu or panitumumab-MCP-$^{177}$Lu to EGFR positive MDA-468 human breast cancer cells ......................................................... 88
Figure 2-5. In vitro stability of the RIVCs against transchelation to EDTA and in human plasma at 37°C ......................................................................................................... 90
Figure 2-6. In vivo stability of the $^{111}$In-RICs in NOD/SCID mice up to 72 h p.i. ................. 92
Figure 2-7. Tumour and normal tissue uptake with/without preinjection of 10 μg unlabeled panitumumab in NOD-scid mice bearing s.c. PANC-1 xenografts at 72 h p.i. of panitumumab-DOTA-$^{111}$In or panitumumab-MCP-$^{111}$In ................................................. 94
Figure 2-8. SPECT/CT images of NOD-scid mice with s.c. PANC-1 xenografts at 72 h p.i. of 37 MBq of panitumumab-DOTA-\(^{111}\)In or panitumumab-MCP-\(^{111}\)In without or with preinjection of unlabeled panitumumab or non-specific RICs........................................................................................................... 96

Figure 3-1. Clonogenic survival of PANC-1 cells exposed to panitumumab-MCP-\(^{177}\)Lu (0.3-1.2 MBq, 2.5 nmol/L) and density of \(\gamma\)-H2AX foci induced in the nucleus of PANC-1 cells. ....... 114

Figure 3-2. Subcellular distribution in PANC-1 cells after incubation with panitumumab-MCP-\(^{177}\)Lu in the absence or presence of unlabeled panitumumab or with non-specific hIgG-MCP-\(^{177}\)Lu. ......................................................................................................................................... 116

Figure 3-3. Hematology and serum biochemistry analysis of NRG mice administered 6.0 MBq (10 \(\mu\)g) of panitumumab-MCP-\(^{177}\)Lu or normal saline.................................................................................. 120

Figure 3-4. Body weight index and tumour growth index for tumour bearing NRG mice at different times post i.v. injection of 6.0 MBq (10 \(\mu\)g) of panitumumab-MCP-\(^{177}\)Lu or non-specific hIgG-MCP-\(^{177}\)Lu, unlabeled panitumumab (10 \(\mu\)g) or normal saline..................... 122

Figure 3-5. Radioactivity (MBq) in selected source organs at 24, 72, 120 and 168 h post i.v. injection of 6.0 MBq (10 \(\mu\)g) of panitumumab-MCP-\(^{177}\)Lu in NRG mice .................................................. 124

Figure 3-6. Tumour and normal organ biodistribution of panitumumab-MCP-\(^{177}\)Lu (MBq/g) in NRG mice with s.c. PANC-1 human pancreatic cancer xenografts at selected times up to 168 h post-injection........................................................................................................................................ 125

Figure 4-1. Clonogenic survival of PANC-1 cells exposed to increasing radioactivity (0.3 MBq, 0.6 MBq or 1.2 MBq) of panitumumab-DOTA-\(^{177}\)Lu, panitumumab-MCP-\(^{111}\)In or panitumumab-DOTA-\(^{111}\)In (2.5 nmol/L) or to unlabeled panitumumab-DOTA.................................................. 145

Figure 4-2. Assessment of DNA DSBs in PANC-1 cells after incubation with 0.3 MBq, 0.6 MBq or 1.2 MBq of \(^{177}\)Lu-labeled panitumumab-DOTA or \(^{111}\)In-labeled panitumumab-DOTA or panitumumab-MCP................................................................. 147

Figure 4-3. Subcellular distribution in PANC-1 cells after incubation with panitumumab-DOTA-\(^{177}\)Lu with or without excess unlabeled panitumumab or with hIgG-DOTA-\(^{177}\)Lu...... 149

Figure 4-4. Hematology and serum biochemistry analyses of NOD/SCID mice at 14 d after i.v. injection of 10.0 MBq of panitumumab-DOTA-\(^{111}\)In, panitumumab-MCP-\(^{111}\)In or normal saline and NRG mice administered 6.0 MBq of panitumumab-DOTA-\(^{177}\)Lu.................................................. 154

Figure 4-5. Tumour growth index and body weight index in NOD/SCID mice with s.c. PANC-1 xenografts receiving three amounts (10.0 MBq; 10 \(\mu\)g) of panitumumab-DOTA-\(^{111}\)In, panitumumab-MCP-\(^{111}\)In, unlabeled panitumumab (10 \(\mu\)g) or normal saline separated by 3 weeks or in NRG mice with s.c. PANC-1 human PnCa xenografts receiving a single injection of panitumumab-DOTA-\(^{177}\)Lu (6.0 MBq; 10 \(\mu\)g). .................................................................................................................. 156
Figure 4-6. Radioactivity (MBq) in selected organs at 24, 72, 120 and 168 h post i.v. injection of a single amount (6 MBq; 10 μg) of panitumumab-DOTA-177Lu in NRG mice or panitumumab-DOTA-111In (10 MBq; 10 μg) or panitumumab-MCP-111In (10 MBq; 10 μg) in NOD/SCID mice.

Figure S1. Clonogenic survival of PANC-1 cells exposed to 0.3, 0.6 or 1.2 MBq of 177Lu- or 111In-labeled panitumumab-MCP, 177Lu- or 111In-labeled panitumumab-DOTA, or to unlabeled immunoconjugates.

Figure S2. Assessment of unrepaired DNA DSBs in PACN-1 cells exposed for 16 h to 0.3, 0.6 or 1.2 MBq 177Lu- or 111In-labeled panitumumab-MCP, 177Lu- or 111In-labeled panitumumab-DOTA, or to unlabeled immunoconjugates.

Figure S3. Hematology and serum biochemistry analyses of NOD/SCID mice at 14 d after i.v. injection of 10.0 MBq (10 μg) of panitumumab-MCP-111In, panitumumab-DOTA-111In or normal saline and NRG mice administered with 6.0 MBq (10 μg) of panitumumab-MCP-177Lu, panitumumab-DOTA-177Lu or normal saline.

Figure S4. Body weight index and tumour growth index (BWI) in NRG mice with s.c. PANC-1 xenografts receiving single amount (6.0 MBq; 10 μg) of panitumumab-MCP-177Lu, panitumumab-DOTA-177Lu or non-specific hIgG-MCP-177Lu or in mice receiving unlabeled panitumumab (10 μg) or normal saline.

Figure S5. Body weight index and tumour growth index (BWI) in NRG mice with s.c. PANC-1 xenografts receiving three amounts (10.0 MBq; 10 μg) of panitumumab-MCP-111In, panitumumab-DOTA-111In or non-specific hIgG-MCP-111In separated by 3 weeks or in mice receiving unlabeled panitumumab (10 μg) or normal saline.

Figure S6. The expression of EGFR, CD31 and α-SMA on PANC-1 xenografts obtained from NOD/SCID mice and NRG mice.
# List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Auger electron</td>
<td>AE</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine transaminase</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>Bq</td>
<td>Becquerel</td>
</tr>
<tr>
<td>BSO</td>
<td>Bismuth germanate</td>
</tr>
<tr>
<td>BWI</td>
<td>Body weight index</td>
</tr>
<tr>
<td>$^{11}$C</td>
<td>Carbon-11</td>
</tr>
<tr>
<td>CA19-9</td>
<td>Carbohydrate antigen 19-9</td>
</tr>
<tr>
<td>CBC</td>
<td>Complete blood count</td>
</tr>
<tr>
<td>$^{64}$Cu</td>
<td>Copper-64</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>Cyclin-dependent kinase inhibitor 2A</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase 2</td>
</tr>
<tr>
<td>cps/Bq</td>
<td>Counting rate per unit radioactivity</td>
</tr>
<tr>
<td>Cr</td>
<td>Creatinine</td>
</tr>
<tr>
<td>Cs</td>
<td>Cell survival</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CTCs</td>
<td>Circulating tumour cells</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOTA</td>
<td>1,4,7,10-tetraazacyclododecane tetraacetic acid</td>
</tr>
<tr>
<td>DOTA-NHS-ester</td>
<td>1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid mono-N-hydroxysuccinimide ester</td>
</tr>
<tr>
<td>DOTA-TATE</td>
<td>DOTA0-Tyr3-Octreotate</td>
</tr>
<tr>
<td>dps</td>
<td>Disintegration per second</td>
</tr>
<tr>
<td>DSB</td>
<td>DNA double strand breaks</td>
</tr>
<tr>
<td>dTMP</td>
<td>Thymidine monophosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>dUMP</td>
<td>Methylates deoxyuridine monophosphate</td>
</tr>
<tr>
<td>EC</td>
<td>Electron captures</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced permeability and retention</td>
</tr>
<tr>
<td>EUS</td>
<td>Endoscopic ultrasound</td>
</tr>
</tbody>
</table>
EUS-FNA  Endoscopic Ultrasound Guided Fine Needle Aspiration

eV  Electron volt

$^{18}$F  Flourine-18

$^{18}$F-FAZA  $^{18}$F-fluorooazomycin arabinoside

$^{18}$F-FDG  18-fluorodeoxyglucose

$^{18}$F-FLT  3'-deoxy-3'-[18F] fluorothymidine

FBS  Fetal bovine serum

FDA  Food and Drug Administration

FGF  Fibroblast growth factor

FOV  Field of view

FWHM  Full-width-at-half-maximum

67Ga  Gallium-67

68Ga  Gallium-68

GSO[CE]  Cerium-doped gadolinium oxyorthosilicate

Gy  Gray

HACA  Human anti-chimeric antibodies

HASA  Human anti-sheep antibodies

Hb  Hemoglobin

Hcl  Hydrogen chloride

Hct  Hematocrit

HPLC  High performance liquid chromatography

HSG  Histamine-succinyl-glycine

HyNic-MCP  Hydrazino-nicotinamide Metal Chelating Polymer

I.v.  Intravenous

$^{111}$In  Indium-111

$^{123}$I  Iodine-123

$^{131}$I  Iodine-131

Ic  Internal conversion

ID/g  Injected dose per gram

IGF1R  Insulin-like Growth Factor 1 receptor

IgG  Immunoglobulin G

ITLC  Instant thin layer chromatography

$^{17}$Lu  Lutetium-177

LET  Linear Energy Transfer

LSO[Ce]  Cerium-doped lutetium oxyorthosilicate

LYSO[CE]  Cerium-doped lutetium-yttrium oxyorthosilicate

mAb  Monoclonal Antibody

MAP  Mitogen-activated protein

MBq  Mega Becquerel

MCNP  Monte Carlo N-Particle
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDCT</td>
<td>Multi-detector computerized tomography</td>
</tr>
<tr>
<td>MEK</td>
<td>Extracellular Kinase</td>
</tr>
<tr>
<td>MMPI</td>
<td>Matrix Metalloproteinases</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum tolerated dose</td>
</tr>
<tr>
<td>MUC-1</td>
<td>Mucin-1</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut off</td>
</tr>
<tr>
<td>NaHCO3</td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td>NaI[Tl]</td>
<td>Sodium iodide activated with thallium</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor-kB</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localizing sequence peptide</td>
</tr>
<tr>
<td>NTR</td>
<td>Nitroreductase enzyme</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>p.i.</td>
<td>Post injection</td>
</tr>
<tr>
<td>PAm</td>
<td>Polyacrylamide</td>
</tr>
<tr>
<td>PanIN</td>
<td>Pancreatic intraepithelial neoplasia</td>
</tr>
<tr>
<td>PAsp</td>
<td>Polyaspartamide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell death-1</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PE</td>
<td>Plating efficiency</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PGLu</td>
<td>Polyglutamidate</td>
</tr>
<tr>
<td>PI-3K/AKT</td>
<td>Phosphoinositide 3-kinase-Akt</td>
</tr>
<tr>
<td>PL</td>
<td>Polylsine</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
</tr>
<tr>
<td>PnCa</td>
<td>Pancreatic Cancer</td>
</tr>
<tr>
<td>PNETs</td>
<td>Pancreatic neuroendocrine tumours</td>
</tr>
<tr>
<td>PSMA</td>
<td>Prostate specific membrane antigen</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RIT</td>
<td>Radioimmunotherapy</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S.c</td>
<td>Subcutaneously</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDF</td>
<td>Stromal derived factor</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion column</td>
</tr>
<tr>
<td>SF</td>
<td>Survival Fraction</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic Hedgehog</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SMA</td>
<td>Superior mesenteric artery</td>
</tr>
<tr>
<td>SMV</td>
<td>Superior mesenteric vein</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted protein-acid rich in cysteine</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single-photon emission computed tomography</td>
</tr>
<tr>
<td>$^{99m}$Tc</td>
<td>Tecnitium-99m</td>
</tr>
<tr>
<td>TCO</td>
<td>Trans-cyclooctene</td>
</tr>
<tr>
<td>TGFβ-1</td>
<td>Transforming growth factor beta 1</td>
</tr>
<tr>
<td>TGI</td>
<td>Tumour growth index</td>
</tr>
<tr>
<td>TS</td>
<td>Thymidylate synthase</td>
</tr>
<tr>
<td>Tz</td>
<td>Tetrazine</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
</tr>
<tr>
<td>$^{89}$Zr</td>
<td>Zirconium-89</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction
1. Introduction

1.1 Current status in the detection, diagnosis and treatment of pancreatic cancer

1.1.1 Pancreatic cancer epidemiology and etiology

Pancreatic cancer (PnCa) is 12th most commonly diagnosed cancer in Canada (1) but is the fourth most common cause of cancer death in Canada and the United States with its incidence almost equivalent to mortality and an overall 5-year survival rate lower than 5% (2). According to Canadian cancer statistics in 2017, 5,500 Canadians were diagnosed with PnCa and 4,800 died of this disease. Less than 50% of patients with PnCa survive beyond 3.9 months. The high mortality relative to incidence reflects the poor prognosis of PnCa. The complex pathophysiology, late stage diagnosis in more than 60% of cases and unresponsiveness to radiation and chemotherapies are major barriers for treatment of this disease (3). Given the limited improvements in PnCa prevention, detection and treatment, especially relative to the other major cancers, PnCa is expected to become the third leading cause of cancer-related death in the coming years. By 2030, the leading causes of cancer-related death are projected to be lung, liver, and pancreas for men, and lung, breast, and pancreas for women (4).

1.1.2 Detection, diagnosis and staging of PnCa

PnCa is a group of heterogeneous diseases and includes cancer of the endocrine pancreas (islet cell carcinoma, neuroendocrine carcinoma and carcinoid tumours) and exocrine (pancreatic ductal adenocarcinoma and acinar) pancreas. Pancreatic neuroendocrine tumours (PNETs) account for about 6% of all PnCa (5). This disease develops from the abnormal growth of neuroendocrine cells that release hormones which may result in tumour-associated adverse effects in patients. PNETs might be benign or malignant and they grow slower than exocrine tumours. Depending on hormone production this disease can be functional or nonfunctional. Functional types cause hormone-related symptoms, however the majority of PNETs are nonfunctional resulting in late diagnosis.

Acinar cell carcinoma is a very rare form of pancreatic exocrine tumour which overproduces measurable pancreatic lipase in the blood. Among pancreatic cancer pathologies, pancreatic ductal adenocarcinoma (PDAC) accounts for approximately 90% of all cases (3). Pancreatic
adenocarcinoma is defined as neoplasia of epithelial cells lining the pancreatic duct (pancreatic intraepithelial neoplasia, PanIN) and has glandular origin, glandular characteristics, or both.

The stage of the disease which describes the size and location at the time of diagnosis aids in selecting the appropriate treatment for the patient. The TNM system (Table 1-1) is the most widely used system for cancer staging based on 3 factors:

- **T**: Size of the primary tumour and whether it has grown outside the pancreas.
  - TX: Primary tumour cannot be assessed
  - T0: No evidence of primary tumour
  - Tis: Carcinoma in situ
  - T1: Tumour limited to the pancreas, ≤2 cm in greatest dimension
  - T2: Tumour limited to the pancreas, >2 cm in greatest dimension
  - T3: Tumour extends beyond the pancreas but without involvement of the celiac axis or the superior mesenteric artery
  - T4: Tumour involves the celiac axis or the superior mesenteric artery
    (unresectable primary tumour)

- **N**: Spread to regional lymph nodes.
  - NX: Regional lymph nodes cannot be assessed
  - N0: No regional lymph node metastasis
  - N1: Regional lymph node metastasis

- **M**: Metastasise to other organs. The most common sites of PnCa spread are the liver, peritoneum and lungs (6).
  - M0: No distant metastasis
  - M1: Distant metastasis
Table 1-1. TNM staging system for PnCa.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Stage grouping</th>
<th>Pancreas</th>
<th>Spread to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Inside</td>
<td>*Outside</td>
</tr>
<tr>
<td>0</td>
<td>Tis, N0, M0</td>
<td>Involvement of the top layers of pancreatic duct cells (pancreatic carcinoma in situ or PanIn III).</td>
<td></td>
</tr>
<tr>
<td>IA</td>
<td>T1, N0, M0</td>
<td>Tumour ≤2 cm (T1)</td>
<td></td>
</tr>
<tr>
<td>IB</td>
<td>T2, N0, M0</td>
<td>Tumour &gt; 2 cm (T2)</td>
<td></td>
</tr>
<tr>
<td>IIA</td>
<td>T3, N0, M0</td>
<td>Yes</td>
<td>No (T3)</td>
</tr>
<tr>
<td>IIB</td>
<td>T1-T3, N1, M0</td>
<td>The tumour is either confined to the pancreas or growing outside the pancreas</td>
<td>No (T3)</td>
</tr>
<tr>
<td>III</td>
<td>T4, Any N, M0</td>
<td>Yes</td>
<td>May be (T4)</td>
</tr>
<tr>
<td>IV</td>
<td>Any T, Any N, M1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Outside means local invasion in this table.
1.2 PnCa biology

PnCa is characterized by having an extremely compact, dense and poorly vascularized stroma which is responsible for limited drug delivery to cancer cells (7), which might be the main reason for the failure of most new treatments evaluated in clinical trials. Generally, PnCa is composed of tumour stroma, PnCa cells, and PnCa stem cells. While numerically small, PnCa stem cells are considered to be resistant to chemotherapy and radiation therapy and their survival is likely responsible for recurrence following treatment (8).

1.2.1 The genomic landscape of PnCa

Genetic analysis of PnCa has revealed that 67% to 100% of the tumours are genetically altered (9). A PnCa cell may carry more than 60 genetic alterations (Figure. 1-1) which can be grouped in 12 core signaling pathways (9).
Figure 1-1. Signaling pathways with mutation in PnCa [Adapted from Jones S (10)].
Mutations in KRAS, p16/cyclin-dependent kinase inhibitor 2A (CDKN21), TP53 and SMAD4/DPC4 drives clonal expansion by conferring a selective growth advantage on PnCa cells. This signature molecular profile which is frequently found in PanIN triggers neoplastic transformation and tumour progression (11, 12). Generation of PnCa from pancreatic inflammation is stimulated by Hedgehog signaling, Notch signaling, and cyclooxygenase 2 (COX-2) (13-15). COX-2 mediates prostaglandin synthesis which then triggers cell proliferation and cytokine synthesis. Pro-inflammatory cytokines and reactive oxygen species (ROS) associated with extensive inflammation activate apoptosis as a cellular protective mechanism as well as proliferation to rebuild the loss of tissue. Enhanced proliferation in the presence of ROS and other potential mutagens lead to accumulation of growth promoting mutations and confer a selective growth advantage to individual cell clones (16). Nuclear factor-kB (NF-kB), the prototypical proinflammatory signaling pathway is also considered as a driver of tumourogenesis from inflammation (17). Important cancer-associated genes, such as cmyc, jun B Cyclin D1, TP53 and vascular endothelial growth factor (VEGF) are under the control of this transcription factor (16).

1.2.2 PnCa stem cells

In early stage PnCa, stem cells are the principal tumour compartment and numerically decrease as the disease advances. The most important phenotypic characteristics of these cells are the capacity for self-renewal and asymmetric division. PnCa stem cells in primary tumours is associated with shorter overall survival, resistance to gemcitabine, enhanced metastatic potential and tumour heterogeneity (18, 19). PnCa stem cells are very plastic with transition among different states including from epithelial to mesenchymal states which may be involved in the metastatic spread of PnCa (20, 21). The expression of unique targets in PnCa stem cells led to many targeted therapeutic studies. Most of these targets belong to pathways such as Hedgehog, Wnt and Notch, apoptotic pathway targets such as DR5 and nodal-activin Alk4/7 pathway (22-25).

1.2.3 The tumour microenvironment

A characteristic of PnCa is the formation of a dense stroma, termed a desmoplastic reaction (26, 27). The pancreatic stellate cells (PSCs) (also known as myofibroblasts) are the principle source
of fibrosis and interact closely with PnCa cells to form the tumour (28). Growth factors such as transforming growth factor beta 1 (TGFβ-1), platelet derived growth factor (PDGF), and fibroblast growth factor (FGF), activates PSCs for secretion of different components of the extracellular matrix including collagen and matrix-metalloproteinases through which they regulate the reabsorption and turnover of the stroma (29). PSCs are also responsible for the poor vascularization characteristics of PnCa (29, 30). Secreted protein-acid rich in cysteine (SPARC), is a calcium-binding protein and is highly expressed in PSCs and stromal fibroblasts immediately adjacent to PnCa cells, with an important role in promoting epithelial-to-mesenchymal transition and invasion through matrix metalloprotease expression (31). It is hypothesized that SPARC actively binds the albumin in nab-paclitaxel which is an albumin coated nanoparticle of paclitaxel used for treatment of PnCa and further concentrates the drug in the tumour contributing to stromal collapse (32). In patient derived PnCa xenografts in mice, nab-paclitaxel eliminated the stroma, increasing the delivery of gemcitabine and was associated with high antitumour activity (21). SPARC overexpressing PSCs are also responsible for radioprotection of PnCa cells through β1-integrin signaling (33) and by induction of hypoxia by producing abundant collagen in the tumour stroma (21).

In addition to being a mechanical barrier, the stroma is a dynamic compartment involved in the process of tumour formation, progression, invasion, and metastasis (26, 27). Among the proteins expressed by stromal cells, Cox-2, PDGF receptor, VEGF, stromal derived factor (SDF), chemokines, integrins, SPARC and hedgehog pathway elements, have been associated with a worse prognosis and resistance to treatment (34). Preclinical studies have shown that the hedgehog inhibitors such as Saridegib (IPI-926) or enzymatic targeting of hyaluronic acid in the stroma using PEGPH20 (pegvorhyaluronidase alfa) disrupt the stroma, increase vascular supply and improve drug delivery (35-37). However the results obtained from the clinical trials haven’t been promising thus far. The tumour microenvironment of PnCa is also immunosuppressive inhibiting antitumour immunity (21). The cell surface molecule CD40 is a member of the tumour necrosis factor receptor superfamily and is broadly expressed by immune cells, in particular B cells, dendritic cells (DC), and monocytes, as well as other normal cells and some malignant cells (38-40). Clinical studies have shown that treatment of patients with surgically incurable PnCa using agonist CD40 antibody combined with gemcitabine led to tumour regression (40).
Activation of CD40 in T cells stimulates the infiltration of tumour macrophages that deplete the cancer stroma (40).

1.2.4 Circulating tumour cells

Circulating tumour cells (CTCs) are tumour cells that have acquired the ability to enter the circulatory system. Frequent metastases of PnCa to the liver, lung and skeletal system reflects the ability of these cells to detach from the primary tumour, and travel through the circulation to distant organs, where they extravasate to establish metastases. In early stages of tumour formation, tumourmalignant cells may be passively shed from the primary tumour in large numbers and enter the blood circulation (41, 42). Furthermore transition of epithelial cells to a mesenchymal phenotype and acquiring corresponding characteristics including motility, invasiveness and resistance to apoptosis promotes detachment from the primary tumour (43). There are different ways of CTC dissemination (44, 45) and the different sites of metastases that also shed tumour cells (46, 47) results in considerable heterogeneity within the CTC population. Most CTCs do not have the ability to form distant tumours, and only 0.01% of CTCs survive (48, 49) and have the characteristics of cancer stem cells (50).

1.3 Treatment of pancreatic cancer

PnCa exhibits an aggressive biological phenotype characterized by early invasion of surrounding structures and rapid metastatic spread and resistance to radiation therapy and/or chemotherapy (51). More than 80% of PnCa patients present with unresectable disease and one third of these patients have locally advanced PDAC while the remainder have distant metastases (52). Chemotherapy remains the only option for locally advanced or metastatic disease. Unfortunately the response of patients is quite limited to chemotherapy due to both intrinsic and acquired resistance to chemotherapeutic agents, therefore there is an urgent need for novel therapeutic strategies.

1.3.1 Surgery

The Whipple procedure, or Kausch-Whipple procedure also known as pancreaticoduodenectomy or pancreatoduodenectomy, is performed to remove resectable tumours of the pancreas (53). However, only 20% of patients are eligible for surgery (54) depending on the presence or
absence of a fat layer creating a barrier between the tumour and major vessels (55) the extent of local tumour invasion, anatomical adjacency to, or involvement of blood vessels (56). Due to the shared blood supply of organs in the proximal gastrointestinal system, surgical removal of the head of the pancreas, also necessitates removal of the duodenum, proximal jejunum, gallbladder, and, occasionally, part of the stomach. The success of the Whipple procedure also depends on the surgical expertise and general performance status of patients (54).

Computed tomography (CT) is usually used to determine whether or not surgical resection is possible, however sometimes it only becomes apparent during the surgery that the tumour cannot be removed without damaging vital tissues. Following surgery the existence of residual cancer cells at the margins of removed tissue is examined microscopically by a pathologist (56). Unfortunately the presence of microscopic deposits of cancer stem cells which may continue to repopulate the tumour and aid in its invasion are not evident microscopically (34, 57). Treatment of a post-operative complications which is not caused by the cancer itself is an issue for 30–45% of patients. Difficulty in emptying the stomach is the most common complication of surgery (55).

1.3.2 Chemotherapy

Patients who are not eligible for surgery receive chemotherapy in order to extend life or improve its quality (55). For borderline resectable tumours neoadjuvant chemotherapy or chemoradiotherapy may be used to reduce the size of tumour to enable surgery. After a recovery period of one to two months following surgery, adjuvant chemotherapy is offered to patients. There are currently five Food and Drug Administration (FDA) approved chemotherapy drugs for the treatment of PnCa: fluorouracil (5-FU), gemcitabine (Gemzar), FOLFIRINOX, albumin-bound paclitaxel (ABRAXANE), and irinotecan liposome injection (ONIVYDE).

1.3.2.1 5-Fluorouracil

5-Fluorouracil (5-FU) principally acts as a thymidylate synthase (TS) inhibitor. Thymidylate synthase methylates deoxyuridine monophosphate (dUMP) to form thymidine monophosphate (dTMP) (Figure 1-2.). Interrupting the action of this enzyme blocks the synthesis of the pyrimidine thymidine, which is a nucleoside required for DNA replication. Depletion of dTMP in rapidly dividing cancer cells causes cell death.
Figure 1-2. Methylation of deoxyuridine monophosphate (dUMP) to produce deoxythymidine monophosphate (dTMP) by thymidylate synthase (TS). 5-FU inhibits TS.
The chemotherapy drugs most commonly used in conjunction with radiation therapy are 5-FU and gemcitabine. 5-FU is used most often since there is more experience using this drug in combination with radiation and there are fewer side effects (58).

1.3.2.2 Gemcitabine

Gemcitabine (Gemzar) is a nucleoside analog of pyrimidine in which the hydrogen atoms on the 2' carbon of deoxycytidine are replaced by fluorine atoms (Figure.1-3). Gemcitabine was approved by the U.S. FDA for treatment of PnCa in 1997 after it was shown to increase the median survival of patients with advanced PnCa by 5 weeks compared to 5-FU which was the standard treatment for unresectable PnCa at the time (59).
Figure 1-3. The chemical structure of gemcitabine.
In the clinical trial conducted by Burris et al. 126 patients with advanced symptomatic PnCa were randomized to receive either gemcitabine 1,000 mg/m\(^2\) weekly \(\times\) 7 weeks followed by 1 week of rest, then weekly \(\times\) 3 weeks for 4 cycles thereafter (63 patients), or to 5-FU 600 mg/m\(^2\) once weekly (63 patients). The median survival was 5.65 and 4.41 months for gemcitabine-treated and 5-FU-treated patients, respectively (\(P = 0.0025\)). The proportion of patients surviving at 12 months was 18% for patients receiving gemcitabine and 2% for 5-FU. Treatment with gemcitabine was well tolerated (59).

### 1.3.2.3 FOLFIRINOX

FOLFIRINOX was recently approved as a more effective chemotherapy regimen than monotherapy with gemcitabine for advanced and metastatic PnCa. This regimen is a combination of four drugs that is effective but also associated with significant normal tissue toxicity:

- 5-FU
- Folinic acid (leucovorin): a vitamin B derivative that reduces the side effects of 5-FU.
- Irinotecan (Camptosar): a topoisomerase inhibitor, which prevents DNA from uncoiling and duplicating.
- Oxaliplatin (Eloxatin): a antineoplastic agent, which inhibits DNA repair and/or DNA synthesis (60).

A randomized phase III clinical trial of 342 patients with metastatic PnCa conducted by Conroy et al in 2011 (61) showed that patients on the FOLFIRINOX treatment (oxaliplatin, 85 mg/m\(^2\) of body-surface area; irinotecan, 180 mg/m\(^2\); leucovorin, 400 mg/m\(^2\); and 5-FU, 400 mg/m\(^2\) given as a bolus followed by 2400 mg/m\(^2\) given as a 46-hour continuous infusion, every 2 weeks) lived 4 months longer than patients receiving the standard gemcitabine treatment (1000 mg/m\(^2\) weekly for 7 of 8 weeks and then weekly for 3 of 4 weeks). The median overall survival was 11.1 months in the FOLFIRINOX group as compared with 6.8 months in the gemcitabine group and median progression-free survival was 6.4 months in the FOLFIRINOX group and 3.3 months in the gemcitabine group (hazard ratio for disease progression, 0.47; 95% CI, 0.37 to 0.59; \(P<0.001\)).
Due to the substantial side effects, FOLFIRINOX is only suitable for patients with good performance status (62) meaning there is still much needed advancement in the systemic treatment of PnCa.

1.3.2.4 Protein-bound paclitaxel (nab-paclitaxel)

In 2013, positive findings from the phase III MPACT trial led to the U.S. FDA approval of nanoparticle albumin-bound paclitaxel (nab-paclitaxel or Abraxane) combined with gemcitabine for treating late-stage PnCa as a less toxic (although less effective) alternative to FOLFIRINOX. Nab-paclitaxel is a colloidal suspension of 130 nm particles homogenized in human serum albumin that incorporate paclitaxel with better bioavailability compared to solvent-based paclitaxel (63) (Figure 1-4.). Nab-paclitaxel binds to the albumin receptor (gp60) as well as SPARC antigen expressed on cancer cells (64, 65). SPARC is a calcium-binding protein and highly expressed in stromal fibroblasts immediately adjacent to PnCa cells, with an important role in promoting epithelial-to-mesenchymal transition and invasion through matrix metalloprotease expression (31).
Figure 1-4. Nab-paclitaxel or Abraxane. The formulation is prepared by high-pressure homogenization of paclitaxel in the presence of serum albumin into a nanoparticle colloidal suspension. The human albumin-stabilized paclitaxel particles have an average size of 130 nm.
A total of 861 patients with metastatic PnCa were randomly assigned to receive nab-paclitaxel plus gemcitabine or gemcitabine alone. The median overall survival (OS) was significantly longer for patients treated with nab-paclitaxel plus gemcitabine vs gemcitabine alone (8.7 vs 6.6 months). Long-term (>3-year) survivors were identified in the nab-paclitaxel plus gemcitabine arm only (4%) (63).

1.3.2.5 Irinotecan liposome injection (ONIVYDE)

Irinotecan liposome injection (ONIVYDE) in combination with 5-FU and leucovorin, was approved in October 2015 as treatment for metastatic PnCa that has progressed following treatment with gemcitabine based therapy (Figure 1-5). The phase III NAPOLI-1 trial of this regimen achieved a substantial improvement in 12-month OS compared to 5-FU and leucovorin alone in patients with metastatic PnCa who had received prior gemcitabine chemotherapy (64).
Figure 1-5. Irinotecan liposome injection (ONIVYDE). Unilamellar lipid bilayer vesicles of approximately 110 nm in diameter encapsulate 80,000 molecules of irinotecan in a gelated or precipitated state, as sucrosofate salt. These liposomes release irinotecan slowly over time.
Currently, patients able to manage the side-effects receive FOLFIRINOX or nab-paclitaxel with gemcitabine while those who cannot tolerate the side effects of this regimen receive gemcitabine alone. Patients with metastatic PnCa who don’t respond to gemcitabine receive (ONIVYDE) in combination with 5-FU and leucovorin. Clinical trials are actively being conducted for novel adjuvant therapies, since new therapies introduced in the last few years have only increased survival times by a few months (62).

1.4 Diagnostic imaging modalities for PnCa

Imaging plays an important role, not only for initial diagnosis and staging, but also for determining the resectability and monitoring the effectiveness of treatment of PnCa (65-67). Complete surgical resection of the tumour increases the survival of patients to 12-20 months, however there is a high probability of relapse due to the highly adverse and aggressive nature of PnCa (68, 69). Multi-detector computerized tomography (MDCT) is currently the imaging modality of choice for evaluation of PnCa and determining its resectability (70).

1.4.1 Contrast-enhanced abdominal ultrasound

Contrast-enhanced abdominal ultrasound is a non-invasive and cost-effective imaging modality for patients presenting with jaundice or abdominal pain (70). This method involves the administration of intravenous contrast agents containing microbubbles of nitrogen or perfluorocarbon. Pulses of ultrasound are transmitted into tissue using a probe, the sound echoes off tissue interfaces and are recorded and displayed as an image. Different tissues have varying degrees of sound wave reflection. Drawbacks of ultrasonography include limits on its field of view, body habitus and its dependence on a skilled operator. Ultrasound has a 50%-90% sensitivity for detecting PnCa (71-75).

1.4.2 MDCT

The most important pre-operative examination in patients with suspected PnCa is MDCT owing to its very good spatial resolution and wide anatomic coverage, allowing local and distant disease assessment (76). The capability to assess vascular involvement critically important for predicting tumour resectability (77-83) is a major advantage of this imaging modality. The reported positive
predictive value, sensitivity, and specificity for predicting the resectability of PnCa are 89%, 100%, and 72%, respectively (84). Since MDCT may fail to depict small liver or peritoneum metastases (80), for treatment monitoring MDCT may be used in conjunction with PET/CT (85, 86).

1.4.3 Endoscopic Ultrasound Guided Fine Needle Aspiration (EUS/EUS-FNA)

Endoscopic ultrasound guided fine needle aspiration (UES-FNA) combines imaging and tissue sampling and on occasion collection of pancreatic cyst fluid. EUS-FNA is usually used to further evaluate abnormal findings on CT, MRI or ultrasound. The EUS component involves the insertion of an echoendoscope composed of a thin flexible tubular transducer into the gastrointestinal (GI) tract. FNA uses a specialized needle that can be inserted through the wall of the stomach or intestine into the pancreas allowing for sampling tissues of interest for histological analysis.

As EUS offers excellent visualization of the pancreas from within the duodenum or stomach and can produce high-resolution images of the pancreas, it has been considered one of the most accurate methods for the detection of pancreatic focal lesions, especially in patients with small tumours of 3 cm or less (70, 87, 88). Using EUS for guidance, a biopsy needle can be accurately inserted into the area of interest in the pancreas. EUS-FNA helps to determine the nature of a suspicious lesion, particularly if it may be malignant. EUS is an excellent test for examining an array of pancreatic conditions including pancreatic masses, tumours, cysts, acute and chronic pancreatitis, and autoimmune pancreatitis (89). Absence of an identifiable mass lesion on EUS rules out PnCa with almost 100% certainty (90). EUS may also have a role in preoperative staging of PnCa for determining resectability but MDCT is the most accurate. Portal vein and splenic vein invasion can be visualized with EUS. However, tumour involvement of the superior mesenteric vein (SMV) and the superior mesenteric artery (SMA) are not reliably determined by EUS (91).

1.4.4 MRI

The outstanding soft-tissue contrast of MRI confers an advantage for characterizing pancreatic masses compared to CT. In most cases small tumours, a hypertrophied pancreatic head, isoattenuating PnCa, and focal fatty infiltration of the parenchyma can be depicted by MRI but
not by CT (66). MRI with magnetic resonance cholangiopancreatography (MRCP) makes use of heavily T2-weighted MRI pulse sequences (92). These sequences show high signal in static or slow moving fluids within the gallbladder, biliary ducts and pancreatic duct, with low signal of surrounding tissue. MRCP is a successful technique for delineating the pancreatic ductal system and detecting ductal narrowing due to the presence of a small mass. Comprehensive morphological information on the pancreas parenchyma and the pancreatic duct improve the chance of tumour diagnosis at an early stage (68) and offer a problem-solving tool for patients with non-cancerous pancreatic disease.

1.4.5 Nuclear medicine imaging

Nuclear medicine imaging non-invasively provides functional information at the molecular level that augment our understanding of disease and disease processes by measuring the uptake and turnover of target-specific radiotracers in tissue (93). Nuclear medicine imaging includes positron emission tomography (PET) and single photon emission computed tomography (SPECT). SPECT and PET offer high sensitivity using intravenously administered minimal concentrations of imaging probes in the pico- to nanomolar range (94). Furthermore as opposed to optical and ultrasound imaging with limited detection of signals from deeper tissues, SPECT and PET are able to detect radioactivity from deep tissues. Quantitative information on radiotracer distribution and target expression can be obtained by SPECT and PET imaging. PET imaging with 18-fluorodeoxyglucose ($^{18}$F-FDG) has been used for staging and restaging cancer, detecting recurrence, and treatment response monitoring (95). Clinical SPECT imaging is of great utility for diagnostic purposes such as the localization of primary and metastatic somatostatin receptor-expressing neuroendocrine tumours using $^{111}$In-pentetreotide (Octreoscan™) (96), the identification of bone metastases using $^{99m}$Tc-methylene diphosphonate (MDP) (97), and the staging of pelvic lymphadenectomy or the detection of metastatic lymph node involvement in prostate cancer using $^{111}$In-capromab pentetide (98). The addition of anatomic CT imaging to PET and SPECT functional imaging has further expanded the utility and accuracy of nuclear medicine imaging (93). SPECT and PET imaging will be discussed in the following sections.
In PET, collision of an electron with the positron emitted by radioisotope, results in annihilation of the positron and creation of two anti-parallel 511 KeV $\gamma$-photons. Simultaneous detection of these two $\gamma$-photons within a coincidence-timing window of a few nanoseconds by opposing inorganic scintillation crystal detectors and affixed photomultiplier tubes (PMT) located in a 360-degree ring creates a line of response (LOR) through the point of decay (Figure 1-6). The raw data is collected from many angles around the patient’s body and analysed by the scanner computing unit and store as a list mode data from which the sinogram can be calculated and reconstructed to PET image using filtered back projection or iterative algorithms (99, 100). The scintillation crystals used for detection are usually bismuth germanate (BSO), cerium-doped gadolinium oxyorthosilicate (GSO[Ce]), cerium-doped lutetium oxyorthosilicate (LSO[Ce]) and cerium-doped lutetium-yttrium oxyorthosilicate (LYSO[Ce]) which have greater stopping power for the 511-keV $\gamma$-photons than sodium iodide activated with thallium, (NaI[Tl]) scintillators due to their higher mass density and effective atomic number (101). During a PET scan, several million coincidence events are recorded generating many intersecting LORs providing information on the spatial location of radioactive decays in the body. The reconstruction of the image in a PET system requires crossing many LORs (Figure 1-6B). A LOR can be formed by a true coincidence, which occurs when both photons from an annihilation event are detected, neither photon undergoes any form of interaction prior to detection, and no other event is detected within the coincidence-timing window (102). If any of the photons from an annihilation event scatters and changes its direction prior to detection, the resulting coincidence will be scattered coincidence and represent a wrong LOR. Another type of coincidence is random coincidence that occurs when two photons, not arising from the same annihilation event, impinge the detectors within the coincidence-timing window of the system. Scatter and random coincidences add a background to the true coincidence distribution, decreasing contrast and causing the isotope concentrations to be overestimated (102).
Figure 1-6. PET imaging system and types of coincidences in PET. A) Opposing scintillation detectors and affixed PMTs located in a 360-degree ring detect anti-parallel 511 KeV $\gamma$-photons and create a LOR through the point of decay. Undesired LORs are crossed by the coincidence processing unit prior to image processing. B) True coincidence; photons from an annihilation event are detected without any prior interaction with the medium. Scattered coincidence; one of the photons scatters and changes its direction prior to detection. Random coincidence; simultaneous detection of two photons from different annihilation events by detectors. [Adapted from Langner J (103)].
**Spatial resolution for PET imaging:** Spatial resolution is defined as the ability to clearly delineate two neighbouring sources and is expressed as the full-width-at-half-maximum (FWHM). It is empirically defined as the minimum distance between two points in an image that can be detected by a scanner (104). The spatial resolution of PET is limited by factors such as positron range, photon non-collinearity, detector size, and reconstruction methods (104, 105). Positron range refers to the average distance that a positron travels in a surrounding medium before it encounters an electron and is annihilated (106). This range is directly dependent on the energy of the positron whereby higher energy positrons travel further. As a result, the origin of the γ-photon is not the position of the radionuclide but some distance from it (107). Photon non-collinearity is the angular deviation from the 180° (<0.5° C (108)) trajectories travelled between the two annihilation photons (109) which results in resolution blurring that is dependent on the ring diameter of the detector causing a misplacement of approximately 1 mm for a 50 cm ring diameter and increasing to 2 mm for a 90 cm ring which is used for whole-body scanners (108). The use of smaller detector elements and smaller diameters of detector rings in small animal PET scanners results in spatial resolutions of 0.83 mm (105). The cut-off frequency of a filter defines the frequency above which the noise is eliminated (110). In the filtered back projection reconstruction method a filter with a too high cut off value introduces noise and thus degrades spatial resolution (104).

In small animal PET systems the detector rings in the axial direction have been expanded, therefore due to the large number of detector crystals ranging from 25,600 to 32,448 (111, 112) the sensitivity of the small-animal PET systems is about 3 times that of a conventional PET scanner (112). Examples of positron emitting isotopes used for PET and their properties are listed in Table 1-2.
### Table 1-2. Physical properties of some positron emitting radionuclides used in PET imaging.

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Physical half-life</th>
<th>Maximum positron energy (MeV)</th>
<th>Positron branching ratio (%)</th>
<th>Positron range in water (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon-11 ((^{11})C)</td>
<td>20.4 min</td>
<td>0.96</td>
<td>99</td>
<td>0.4</td>
</tr>
<tr>
<td>Flourine-18 ((^{18})F)</td>
<td>1.83 h</td>
<td>0.64</td>
<td>97</td>
<td>0.2</td>
</tr>
<tr>
<td>Copper-64 ((^{64})Cu)</td>
<td>12.7 h</td>
<td>0.58</td>
<td>19</td>
<td>0.2</td>
</tr>
<tr>
<td>Zirconium-89 ((^{89})Zr)</td>
<td>78.4 h</td>
<td>0.90</td>
<td>23</td>
<td>0.4</td>
</tr>
<tr>
<td>Gallium-68 ((^{68})Ga)</td>
<td>1.14 h</td>
<td>1.90</td>
<td>88</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Adapted from Zanzonico P (101)
**18F-FDG PET imaging:** The increased utilization of glucose by tumours has been attributed to an increased activity of membrane glucose transporters such as GLUT1 (113-115). PET scanning with the radiotracer 18F-FDG relies on the increase rate of glycolysis. Like glucose, FDG is transported by GLUT1 and phosphorylated by hexokinase, however, since FDG-6-phosphate is not a substrate for the next enzyme (phosphohexose isomerase) in the glycolytic pathway, it cannot proceed along the pathway (116). 18F-FDG-6-phosphate is trapped within the tumour cell and imaged by PET. This imaging technique has moved into the clinical realm of diagnosis, staging, and treatment planning (117-119). High accumulation of 18F-FDG in a lesion suggests the presence of metabolically active cancer cells, although infiltration of inflammatory cells may also cause 18F-FDG uptake. False negative PET scans has been reported in patients with elevated blood glucose levels, which interfere with 18F-FDG uptake (91) or small lesions (low detection sensitivity due to the spatial resolution limits of PET). There is still no consensus on whether PET provides information beyond that obtained by contrast-enhanced CT (72) and even the utility of PET for identifying metastases is controversial (91). Mertz et al. studied 35 patients with presumed resectable PDAC with CT, EUS, and 18FDG-PET(120). They showed that sensitivity for the detection of PnCa was higher for EUS (93%) and 18FDG-PET (87%) than for CT (53%). 18FDG-PET diagnosed 7 of 9 cases of proven metastatic disease, whereas CT missed 4 of them. Two of three metastatic liver lesions suspected by CT were indeterminate for metastases but 18FDG-PET confirmed metastases. In another study Wakabayashi et al. compared the sensitivity and accuracy of tumour diagnosis for 18FDG-PET and CT in 53 patients with proven primary PnCa (121). The sensitivity of 18FDG-PET, CT, were 92.5%, 88.7%; respectively. 18FDG-PET was superior to CT in diagnosing distant disease (bone metastasis) but for local staging, the sensitivity of CT was better than that of 18FDG-PET. However recent studies have shown that integration of PET/CT into the diagnostic work-up is superior to conventional imaging (MDCT, CT angiography, EUS) alone for tumour staging, especially related to detection of distant metastases (sensitivity and specificity rates were 89% versus 56% and 100% versus 95%, respectively) (91). Utility of 18F-FDG PET/CT in diagnosis, staging, detection of liver metastasis and assessment of resectability and metabolic response of PnCa has been studied in different clinical trials [reviewed in (122)]. Studies have shown that 18FDG-PET/CT has an advantage in monitoring metabolic response, making it optimal in evaluation of different kinds of treatments and is also a valuable tool to detect suspected recurrence (122).
Since 2011, $^{18}$F-FDG PET/CT has been approved by Cancer Care Ontario for PnCa patients who have potentially resectable tumour (123).

PET imaging using $^{18}$F-fluoroazomycin arabinoside ($^{18}$F-FAZA) has proven effective for stratification of patients with hypoxic PnCa and to estimate perfusion and hypoxic fraction in tumour (124). Owing to its lipophilicity, $^{18}$F-FAZA is diffused through the cell membrane, reduced by nitroreductase enzyme (NTR) trapped in the cell (125). When the cell is well oxygenated, this process is reversible and the tracer can freely flow back into the extracellular environment. 3’-deoxy-3’-[18F] fluorothymidine ($^{18}$F-FLT) is another imaging tracer that is preferentially retained in proliferating cells (126). Intracellular thymidine kinase 1 (TK1), catalyzes the phosphorylation of $^{18}$F-FLT to $^{18}$F-FLT-monophosphate which is trapped in cells because of its negative charge (127, 128). The ability of $^{18}$F-FLT to scan primary PnCa using PET is still under investigation in preclinical and clinical studies (129, 130).

1.4.5.2 Single-photon computed tomography (SPECT)

Single photons emitted by radionuclides such as γ-rays arising from isomeric transition, and X-rays arising from electron capture or internal conversion are detected by a gamma camera which rotates around the patient to generate a series of 2-dimensional images (slices) that are then reconstructed to create a 3-dimensional image (101). The gamma camera (Figure 1-7) consists of a collimator to isolate individual γ-photons directly originating from a source in the patient, a large scintillation crystal [most often NaI(Tl)] which converts γ-photons into light, and photomultiplier tubes (PMTs) to convert the received light into electrons and amplify the output pulses. An X, Y position logic circuit sums up the output from the array of PMTs to produce X and Y pulses that are in direct proportion to the X, Y coordinates of the point of interaction of the γ-rays which results in an image of the radioactivity distribution within the patient. Pulses are further electronically sorted and processed for display (131). Following acquisition of a series of planar images (also called projections) throughout the 360° rotation of the camera around the patient, images are reconstructed by filtered back projection or by iterative algorithms to produce a functional 3-dimensional distribution of a photon emitter radionuclide within the body (132).
Figure 1-7. Composition of the SPECT camera. Photons that pass through the collimators are converted to light in scintillation crystal and subsequently amplified by photomultiplier tubes. The output from the array of photomultiplier tubes is processed by a position logic circuit to produce X and Y pulses that are in direct proportion to the X, Y coordinates of the interaction point of the γ-rays. Pulses are further processed for display by filtered back projection or by iterative algorithms to produce a functional 3-dimensional image using a data analysis computer.
It’s important to mention that collimators reduce processing of scattered or degraded photons. This reduces noise and increases spatial resolution but attenuates the majority of incoming photons and therefore greatly reduces sensitivity (106). Ideally, radionuclides with \( \gamma \)-photon energies between 100-200 keV are most desirable due to the ability to adequately penetrate through tissue and yet be efficiently collimated and also detected by the NaI(Tl) scintillation crystal (101). Examples of single photon emitting radioisotopes used for SPECT imaging are provided in Table 1.3
<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Physical half-life</th>
<th>Energies of imageable X and γ-rays (KeV)</th>
<th>Abundance of imageable X and γ-rays (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indium-111 ((^{111})In)</td>
<td>2.83 d</td>
<td>172</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>247</td>
<td>94</td>
</tr>
<tr>
<td>Tecnitium-99m ((^{99m})Tc)</td>
<td>6.01 h</td>
<td>140</td>
<td>89</td>
</tr>
<tr>
<td>Iodine-123 ((^{123})I)</td>
<td>13.2 h</td>
<td>159</td>
<td>84</td>
</tr>
<tr>
<td>Iodine-131 ((^{131})I)</td>
<td>8.04 d</td>
<td>364</td>
<td>82</td>
</tr>
<tr>
<td>Gallium-67 ((^{67})Ga)</td>
<td>3.26 day</td>
<td>93</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>185</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>16</td>
</tr>
</tbody>
</table>

Adapted from Zanzonico (101)
**Spatial resolution for SPECT imaging:** The most limiting factor for spatial resolution is the diameter of the holes in a collimator. There is often a trade-off between spatial resolution and sensitivity. Smaller collimator hole size results in increased rejection of scattered $\gamma$-photons which increases spatial resolution but reduces the number of $\gamma$ photons reaching the crystal, and therefore reduces sensitivity. Spatial resolution of a clinical SPECT is 5-10 mm (133) which is at least an order of magnitude poorer than that of CT and MRI (101). However, the newest clinical SPECT scanners with multi-pinhole collimators (G-SPECT, MILabs) have increased $\gamma$-photon sensitivity and offer spatial resolutions as low as 3 mm (134, 135). Small-animal SPECT systems using multi-pinhole collimators have been developed that offer spatial resolutions of $<1$ mm FWHM (136). This sub-millimeter resolution is required as organs are several orders of magnitude smaller than humans. Spatial resolution also decreases with increasing distance between the patient and the detector.

**Sensitivity of SPECT vs PET:** The sensitivity of a nuclear imaging system is basically based on its photon-collection efficiency and can be reported as counting rate per unit radioactivity (cps/Bq) in the scanner field of view (FOV) (112). In SPECT, the physical collimators are needed in order to reject photons that are not within a small angular range otherwise the angle of incidence will not be known leading to low geometric efficiencies. In PET, the coincidence detection of photon events negates the need for physical collimation and results in a sensitivity, which is typically 10 to 100-fold higher than SPECT (106). This greater sensitivity results in improved image quality (signal-to-noise ratios) and may accelerate data acquisition time (106). Furthermore, due to the generally shorter half-lives of positron emitters compared to single photon emitters, higher amounts of radioactivity may be injected without increasing radiation doses to normal tissues and consequently, this may also increase sensitivity.

**Temporal resolution of SPECT vs PET:** The ability of an imaging instrument to frequently (over a period of time) capture ‘acceptable’ images of an object in the FOV is defined as temporal resolution. Temporal resolution is important for dynamic studies, in which several images are obtained over a short period of time. Compared to spatial resolution, quantification of temporal resolution is difficult and depends on the reconstruction algorithm being used. Since the temporal resolution is very closely related to the sensitivity of imaging, PET has an advantage over SPECT for dynamic studies.
Quantification of radioactivity in SPECT vs PET: In PET, attenuation depends only on the overall probability of 2 anti-parallel 511-keV photons reaching the opposite detectors. The result is that the attenuation correction factor depends only on the total thickness of the attenuation medium, independent of the depth of the source. Due to the single-photon emission nature of SPECT, attenuation changes depend on the point of emission (106). Accurate quantitation of radioactivity uptake in target tissues remains a challenge for SPECT due to scatter and because it is necessary to know the exact depth within the body where the radioactive decay originated in order to correct for attenuation by overlying tissues. A transmission CT scan can calculate the attenuation correction factors at different positions in the body as well as provide anatomic information for co-registration with SPECT or PET image.

SPECT/CT is not commonly employed for detecting PnCa in the clinic, yet novel tracers may promote its use in the future (137). Spanu et al. conducted a study in 104 patients with non-functioning gastroenteropancreatic (GEP) tumour with overexpression of somatostatin receptor to evaluate the usefulness of $^{111}$In-pentetreotide SPECT/CT in diagnosis and staging of GEP compared to the conventional imaging procedures (CIP), such as CT, MRI and US (138). At both CIP and SPECT/CT, 34/104 patients were classified as no evidence of disease and in 70/104 patients, neoplastic lesions were ascertained. At this stage, there was no significant difference in the sensitivity and accuracy between SPECT/CT (91.4 and 94.2%, respectively) and CIP (71.4 and 80.8%, respectively). Out of 292 ascertained lesions (141 hepatic, 78 abdominal extra-hepatic and 73 extra-abdominal), CIP detected 191/292 (65.4%) lesions in 50 patients, while SPECT/CT detected 244/292 (83.6%) in 64 patient (p<0.0001). No false positive results were found at both SPECT/CT and CIP. SPECT/CT sensitivity and accuracy were higher than CIP in GEP grade 1 and grade 2, neuroendocrine carcinoma and mixed adeno-neuroendocrine carcinoma patients, but significantly only for GEP grade 1. Another study showed that clinical imaging of mesothelin-expressing PnCa in two patients with PDAC using an $^{111}$In-labeled chimeric monoclonal antibody, amatuximab, produced a tumour to background ratio ≥1.2, sufficient for distinguishing between tumour and normal tissue (139).

1.4.6 Summary of clinical roles for imaging in PnCa

In conclusion, MDCT is the preferred initial imaging modality for patients with a high suspicion of PnCa, but MRI may also be used for this purpose (70). The most accurate method for
definitive diagnosis of PnCa is EUS and its combination with CT/MRI can successfully determine tumour resectability. The presence of PnCa on imaging can be confirmed histologically with EUS/EUS-FNA. PET scans can provide valuable information on metastatic disease in PnCa, which changes the stage of the disease and the decision regarding resectability (70). Clinical studies show that PET/CT has higher sensitivity and specificity (97.4% and 91.2%; respectively) compared to CT (85.9% and 67.3%; respectively) for detecting metastatic disease (140).

Small Animal PET and SPECT

PET and SPECT small animal imaging systems provide greater spatial resolution compared to clinical PET and SPECT imaging systems and therefore they can be a translational research tool to develop radiopharmaceuticals for both clinical PET and SPECT as well as radioimmunotherapy. In vivo imaging bridges the gap between in vitro exploratory and in vivo clinical research and facilitates the direct and fast clinical translation of newly developed radiopharmaceuticals. There exists a broad array of preclinical studies evaluating imaging of PnCa using animal imaging systems. High tumour accumulation of AMB8LK antibody targeting ferritin labeled with $^{111}$In in mice with subcutaneous (s.c.) CAPAN-1 xenografts was shown by SPECT/CT at 72 h post-injection (p.i.) with $23.6 \pm 3.9\%$ injected dose per gram (%ID/g) (141).

In another study, overexpression of integrin $\alpha\beta6$, a member of the integrin family, in pancreatic BxPC-3 xenografts was targeted using $^{99m}$Tc-labeled integrin $\alpha\beta6$-targeting peptide ($^{99m}$Tc-HHK) and imaged by SPECT at 0.5 h p.i with an uptake of $0.88 \pm 0.12$ (%ID/g) (142). Liver metastatic BxPC-3 tumour lesions in orthotopic models were clearly detected by SPECT/CT. Targeting epidermal growth factor receptor (EGFR) using panitumumab is another approach to target radioactivity to pancreatic tumour. Panitumumab fab fragments (F(ab')$_2$) produced by proteolytic digestion of panitumumab IgG labeled with $^{64}$Cu using NOTA has been studied for PET imaging of NOD-scid mice engrafted subcutaneously or orthotopically with patient-derived OCIP23 pancreatic tumours, or with s.c. PANC-1 human PnCa xenografts (143). Tumour uptake of the tracer in the mentioned tumour models at 48 h p.i. was $12.0 \pm 0.9\%$ ID/g, $11.8 \pm 0.9\%$ ID/g and $6.1 \pm 1.1\%$ ID/g; respectively and tumour to blood ratios were 5:1 to 9:1 for OCIP23 and 2.4:1 for PANC-1 tumours. All tumour xenografts were clearly imaged by microPET/CT at 24 or 48 h p.i. of $^{64}$Cu-NOTA-panitumumab F(ab')$_2$. Lou et al. developed a bispecific
heterodimer for PET imaging of nude mice bearing BXPC-3 xenograft and orthotopic pancreatic tumours (144). An anti-tissue factor (TF) Fab was conjugated an anti-CD105 Fab, via the bio-orthogonal "click" reaction between tetrazine (Tz) and trans-cyclooctene (TCO) and labeled with $^{64}$Cu. PET imaging of BXPC-3 (TF/CD105(+/+)) xenografts displayed significantly enhanced tumour uptake (28.8 ± 3.2 %ID/g) at 30 h p.i., as compared with monospecific anti-TF Fab or anti-CD105 Fab tracer (12.5 ± 1.4 and 7.1 ± 2.6 %ID/g; respectively) with a tumour to muscle ratio of 75.2 ± 9.4. Tumour uptake of $^{64}$Cu-heterodimer in the orthotopic model was 17.1 ± 4.9 %ID/g at 30 h p.i. with a tumour to muscle ratio of 72.3 ± 46.7.

There is an increasing role for nuclear imaging in oncology. In general, PET and SPECT as non-invasive functional imaging modalities can provide important information on the drug development process (e.g pharmacokinetics and pharmacodynamics of novel therapeutics) by utilizing radiotracers.

### 1.5 Therapeutic targets for PnCa

PnCa treatment continues to be a challenge because of its advanced stage at the time of diagnosis, its aggressive nature, genetic instability and of course limited treatment options available. With identification of various molecular pathways in PnCa tumourigenesis, potential targets for drug development have been pursued with the use of monoclonal antibodies (mAbs) and small-molecule inhibitors (9). Potential targets in pancreatic tumourigenesis that have been studied thus far has been summarized in Figure 1-8.
Figure 1-8. Potential targets in pancreatic tumourigenesis.
Studies have explored some genetic mutations and altered expression patterns, which may serve as potential targets for treatment of PnCa (Table 1-4). Immunohistochemistry studies have shown that 73% of PaCa tumours carried Kirsten rat sarcoma (KRAS) mutation, 74% of them expressed cyclooxygenase-2 (COX-2), 61% had high TOPO 1, and 44% had high SPARC expression.
Table 1-4. Potential molecular targets with frequency of genetic aberration on PnCa and their targeted agents [reviewed in (9)].

<table>
<thead>
<tr>
<th>Target</th>
<th>Frequency of Genetic Aberration</th>
<th>Targeted Agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRAS</td>
<td>&gt;90% (145)</td>
<td>Tipifarnib, salirasib</td>
</tr>
<tr>
<td>VEGF</td>
<td>93% (146)</td>
<td>Bevacizumab, axitinib, Sorafenib, aflibercept</td>
</tr>
<tr>
<td>Mesothelin</td>
<td>90%-100% (147, 148)</td>
<td>SS1P, MORAb-009</td>
</tr>
<tr>
<td>MUC1</td>
<td>90% (149)</td>
<td>Vaccine</td>
</tr>
<tr>
<td>COX-2</td>
<td>67%-90% (150-152)</td>
<td>Celecoxib, apricoxib</td>
</tr>
<tr>
<td>Cholecystokinin/gastrin</td>
<td>95% (152)</td>
<td>Gastrazole</td>
</tr>
<tr>
<td>Glycine-extended gastrin</td>
<td>55% (152)</td>
<td>G17DT</td>
</tr>
<tr>
<td>Amidated gastrin</td>
<td>23% (152)</td>
<td>G17DT</td>
</tr>
<tr>
<td>Notch3</td>
<td>69%-74% (153)</td>
<td>Saridegib (IPI-926)</td>
</tr>
<tr>
<td>Hedgehog</td>
<td>70% (154)</td>
<td>Saridegib (IPI-926)</td>
</tr>
<tr>
<td>EGFR</td>
<td>43%-69% (155, 156)</td>
<td>Erlotinib, cetuximab</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>64% (157)</td>
<td>AMG-479, NVP-AEW541</td>
</tr>
</tbody>
</table>
Although encouraging results have been demonstrated with multiple targeted therapies of PnCa in preclinical studies, only erlotinib, an of epidermal growth factor receptor (EGFR) inhibitor, showed a marginal survival benefit in a phase III clinical trial, when combined with gemcitabine (9). Table 1-5 summarized all phases II/III clinical trials with targeted therapies in PnCa.
Table 1-5. Phase II-III clinical trials with targeted therapies in PnCa [reviewed in (9)].

<table>
<thead>
<tr>
<th>Target</th>
<th>Study Treatment</th>
<th>Trial Phase</th>
<th>No. Patients</th>
<th>PFS</th>
<th>MS</th>
<th>1-y Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GEM + erlotinib (158)</td>
<td>Phase III</td>
<td>285</td>
<td>3.75 mo</td>
<td>6.24 mo</td>
<td>23%</td>
</tr>
<tr>
<td></td>
<td>GEM</td>
<td></td>
<td>284</td>
<td>3.55 mo</td>
<td>5.91 mo</td>
<td>17%</td>
</tr>
<tr>
<td></td>
<td>GEM + cetuximab (159)</td>
<td>Phase II</td>
<td>41</td>
<td>3.8 mo</td>
<td>7.1 mo</td>
<td>31.7%</td>
</tr>
<tr>
<td></td>
<td>GEM + cetuximab (160)</td>
<td>Phase III</td>
<td>372</td>
<td>3.4 mo</td>
<td>6.3 mo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GEM</td>
<td></td>
<td>371</td>
<td>3.0 mo</td>
<td>5.9 mo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VEGF</td>
<td>GEM + BEV (161)</td>
<td>Phase II</td>
<td>52</td>
<td>5.4 mo</td>
<td>8.8 mo</td>
</tr>
<tr>
<td></td>
<td>GEM + BEV (162)</td>
<td>Phase III</td>
<td>279</td>
<td>3.8 mo</td>
<td>5.8 mo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GEM + P</td>
<td></td>
<td>256</td>
<td>2.9 mo</td>
<td>5.9 mo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GEM + erlotinib + BEV (163)</td>
<td>Phase III</td>
<td>306</td>
<td>4.6 mo</td>
<td>7.1 mo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GEM + erlotinib + P</td>
<td></td>
<td>301</td>
<td>3.6 mo</td>
<td>6.0 mo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GEM + sorafenib (164)</td>
<td>Phase II</td>
<td>37</td>
<td>2.9 mo</td>
<td>6.5 mo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sorafenib</td>
<td></td>
<td>15</td>
<td>2.3 mo</td>
<td>4.3 mo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GEM + axitinib (165)</td>
<td>Phase II</td>
<td>69</td>
<td>4.2 mo</td>
<td>6.9 mo</td>
<td>36.8%</td>
</tr>
<tr>
<td></td>
<td>GEM</td>
<td></td>
<td>34</td>
<td>3.7 mo</td>
<td>5.6 mo</td>
<td>23.5%</td>
</tr>
<tr>
<td></td>
<td>GEM + axitinib (166)</td>
<td>Phase III</td>
<td>314</td>
<td>4.4 mo</td>
<td>8.5 mo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GEM</td>
<td></td>
<td>316</td>
<td>4.4 mo</td>
<td>8.3 mo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GEM + aflibercept (167)</td>
<td>Phase III</td>
<td>271</td>
<td>3.7 mo</td>
<td>6.5 mo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GEM + P</td>
<td></td>
<td>275</td>
<td>3.7 mo</td>
<td>7.8 mo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IGF-IR</td>
<td>GEM + ganitumab (168)</td>
<td>Phase II</td>
<td>42</td>
<td>5.1 mo</td>
<td>8.7 mo</td>
</tr>
<tr>
<td></td>
<td>GEM + conatumumab</td>
<td></td>
<td>41</td>
<td>4.0 mo</td>
<td>7.5 mo</td>
<td>20%</td>
</tr>
<tr>
<td></td>
<td>GEM + P</td>
<td></td>
<td>42</td>
<td>2.1 mo</td>
<td>5.9 mo</td>
<td>23%</td>
</tr>
<tr>
<td></td>
<td>MMPI</td>
<td>GEM + marimastat (169)</td>
<td>Phase III</td>
<td>120</td>
<td>92.5 d</td>
<td>165.5 d</td>
</tr>
<tr>
<td></td>
<td>GEM + P</td>
<td></td>
<td>119</td>
<td>96 d</td>
<td>164 d</td>
<td>17%</td>
</tr>
<tr>
<td></td>
<td>BAY 12-9566 (170)</td>
<td>Phase III</td>
<td>138</td>
<td>1.68 mo</td>
<td>3.74 mo</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>GEM</td>
<td></td>
<td>139</td>
<td>3.5 mo</td>
<td>6.59 mo</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td>Gastrin</td>
<td>G17DT responder (171)</td>
<td>Phase II</td>
<td>49</td>
<td>176 d</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G17DT nonresponder</td>
<td></td>
<td>27</td>
<td>63 d</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td></td>
<td>75</td>
<td>83 d</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gastrazole (172)</td>
<td>Phase III</td>
<td>53</td>
<td>7.9 mo</td>
<td>33.3%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-FU</td>
<td></td>
<td>42</td>
<td>4.5 mo</td>
<td>11.1%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MEK-1</td>
<td>Selumetinib (173)</td>
<td>Phase II</td>
<td>37</td>
<td>2.1 mo</td>
<td>5.4 mo</td>
</tr>
<tr>
<td></td>
<td>Capecitabine</td>
<td></td>
<td>32</td>
<td>2.2 mo</td>
<td>5 mo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>COX-2</td>
<td>GEM + cisplatin + celecoxib (174)</td>
<td>Phase II</td>
<td>22</td>
<td>1.8 mo</td>
<td>5.8 mo</td>
</tr>
<tr>
<td></td>
<td>GEM + celecoxib (175)</td>
<td>Phase II</td>
<td>42</td>
<td>5.1 mo</td>
<td>9.1 mo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GEM + irinotecan + celecoxib (176)</td>
<td>Phase II</td>
<td>21</td>
<td>9 mo</td>
<td>18 mo</td>
<td>80%</td>
</tr>
</tbody>
</table>

PFS: Progression-free survival  
MS: median survival  
GEM: gemcitabine  
BEV: bevacizumab  
P: placebo
1.6 Radioimmunotherapy of PnCa by targeting EGFR

EGFR is overexpressed on more than 90% of cases of PnCa in patients (177) and correlates with more advanced disease, poor survival and the presence of metastases (178, 179). EGFR is the first member of the human epidermal growth factor receptor family which also includes the HER2, HER3 and HER4 receptors. Binding of EGF to the EGFR causes receptor homodimerization or heterodimerization with other EGFR family members and initiates mitogenic signaling pathways (180). EGFR-mediated cell signaling plays a major role in promoting tumour proliferation, angiogenesis, metastasis, and evasion of apoptosis (181, 182). Panitumumab or Vectibix is a U.S. FDA and Health Canada-approved fully human IgG2 mAb that binds to the extracellular domain of the EGFR and competitively inhibits EGF binding to EGFR. Panitumumab was initially approved for the treatment of refractory EGFR-expressing metastatic colorectal cancer with disease progression however later on the lack of benefit with anti-EGFR mAbs (panitumumab and cetuximab) in patients who carried KRAS mutations was demonstrated by Amado et al. (182). Since PnCa has the highest incidence of activating KRAS mutations of any cancer at over 90% (183-187), immunotherapy with panitumumab is expected to be ineffective on PnCa cells. However, antibodies can serve as carriers for specific delivery of cytotoxic agents such as radionuclides. For this purpose halogen radionuclides are incorporated directly to antibodies, whereas other radionuclides are complexed to antibodies through chelators. Both preclinical and clinical studies have demonstrated that radioimmunoconjugates (RIC; antibodies labeled with radioisotopes), are more effective than therapy with naked antibodies (187-190). The major reason might be because radioimmunotherapy (RIT) only relies on targeting a cell-surface receptor/antigen to deliver a cytotoxic radionuclide payload to tumours, whereas naked mAbs are intended to block downstream receptor-mediated growth signaling pathways, which may be obviated or circumvented by compensatory upregulation of alternative signaling mechanisms (e.g. KRAS mutation). This is of a great importance for PnCa patients who have tumours that overexpress EGFR but carry KRAS mutation, therefore RIT of PnCa with panitumumab may be a useful alternative strategy to treatment with naked anti-EGFR mAbs.
1.6.1 Types of radiation used in radioimmunotherapy

Radiation can be divided into two classes: i) photon radiation or penetrating radiation such as X-radiation and γ-rays and ii) particle radiation or non-penetrating radiation including alpha particle (α-particle), beta particle (β-particle) and Auger electron. As opposed to photon radiation, particle radiation deposits more energy and this characteristic makes them more efficient for eradicating cancer cells. Basically these particles are characterized based on the amount of energy deposited over the track length of the radiation in tissues (i.e. linear energy transfer: LET). The emission of particles by radioisotope may be accompanied by the emission of gamma photons (γ) allowing SPECT imaging of the delivery of the RIT agents to tumours, dosimetry, and monitoring of therapeutic efficacy. However, γ emissions could contribute to normal tissue toxicity due to their penetrating ability (191, 192).

1.6.1.1 Alpha-particle

An alpha-particle (α-particle) is a helium-4 (\(^{4}\)He) nucleus consisting of two protons and two neutrons which carries a +2 charge. Alpha particles are high energy (4-9 MeV) with high linear energy transfer (LET; 50–230 keV/μm). Moreover, α-particles have a range of only 50–100 μm, which restricts their coverage to 5–10 cell diameters. These properties make α-particles useful for eradicating circulating malignant cells or small clusters of cells. Table 1-4 lists a number of α-emitting radioisotopes of interest for RIT. Some α-emitters (e.g. \(^{225}\)Ac and \(^{225}\)Ra) decay to α-emitting or β-emitting daughter products which creates “radionuclide generator systems”. Such radionuclides deliver a higher radiation absorbed dose to tumours than radionuclides that decay to stable elements or daughter products that emit radiation with lower relative biological effectiveness (RBE) such as β-particles, X- or γ-radiation (190). RBE describes the effectiveness of a particular type of radiation for causing an effect (e.g. cytotoxicity) compared to X- or γ-radiation (191). Although the high RBE provided by the radionuclide generator concept is desirable for RIT, the release and redistribution of the free daughter products to normal tissues has been proposed as a limitation. Since the α-emitting daughters represent different chemistry from their parents atoms they will not remain complexed to chelators substituted onto the mAb. Furthermore the high recoil energy upon decay also promotes their release from the chelators (191). Studies in non-human primates have shown that redistribution of \(^{213}\)Bi released from
\(^{225}\)Ac-labeled anti-CD33 mAb HuM195 caused acute renal dysfunction following deposition of \(\alpha\)-radiation in the kidneys (192-194).

Table 1-6. \(\alpha\)-particle emitters for conjugation to mAbs for RIT of cancer.

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Production method</th>
<th>(\alpha)-particle energy (MeV)</th>
<th>Physical half-life ((t_{1/2P}))</th>
<th>Method for radiolabeling</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{225})Ac</td>
<td>222(^{223})U (\rightarrow) (^{225})Ac</td>
<td>5.8</td>
<td>10 days</td>
<td>Chelation by DOTA or NETA</td>
</tr>
<tr>
<td>(^{211})At</td>
<td>209(^{209})Bi((\alpha,2n))(^{211})At</td>
<td>7.4</td>
<td>7.2 h</td>
<td>Radioastatination</td>
</tr>
<tr>
<td>(^{212})Bi</td>
<td>(^{212})Pb (\rightarrow) (^{212})Bi</td>
<td>6.0</td>
<td>61 min</td>
<td>Chelation by DTPA or DOTA</td>
</tr>
<tr>
<td>(^{213})Bi</td>
<td>(^{225})Ac (\rightarrow) (^{213})Bi</td>
<td>6–8.4</td>
<td>46 min</td>
<td>Chelation by DTPA or DOTA</td>
</tr>
<tr>
<td>(^{212})Pb</td>
<td>(^{224})Ra (\rightarrow) (^{212})Pb</td>
<td>6.0(^{4})</td>
<td>10.6 h</td>
<td>Chelation by TCMC</td>
</tr>
<tr>
<td>(^{227})Th</td>
<td>(^{227})Ac (\rightarrow) (^{227})Th</td>
<td>5.9</td>
<td>18.7 days</td>
<td>Chelation by DOTA</td>
</tr>
</tbody>
</table>

DOTA: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid;
NETA: 4-[2-(bis-carboxymethyl-amino)-ethyl]-7-carboxymethyl-[1,4,7]triazonan-1-yl-acetic acid;
DTPA: diethylenetriaminepentaacetic acid;
\(^{212}\)Pb decays by \(\beta\)-particle emission to \(^{212}\)Bi which emits an \(\beta\)-particle of this energy.
TCMC: 2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraaza-1, 4, 7, 10-tetra-(2-carbamoyl methyl)-cyclododecane.
Considering a RBE of 1 for β-radiation, the RBE range of 3 to 7 for α-particles ranges indicates that at the same radiation absorbed dose biological effects of α-particles are 3 to 7 times greater than for β-radiation (195). Nuclear DNA is the primary target of α-particles for cell death induction. Radiation induced DNA double-strand breaks (DSBs), accumulate phosphorylated histone 2A (gamma-H2AX) at the site, as the first step in recruiting and localizing DNA repair proteins (196). Gamma-H2AX can be detected as a marker of DNA DSBs in vitro by immunofluorescence probing. Hypoxia develops resistance to low LET radiation in cancer cells. As opposed to low LET radiation that requires oxygen free radicals to indirectly damage DNA, the induction of DSBs by α-particles is independent of tissue oxygenation (197, 198). Studies showed that 213Bi-labeled EGFR mAb matuzumab, killed hypoxic CAL 33 squamous cell carcinoma cells as effectively as normoxic cells in vitro (197).

1.6.1.2 Auger electrons

Auger electrons have relatively low energies ranging from a few eV to approximately 100 keV (199). The range of Auger electrons in water is from a fraction of a nanometer to several hundreds of micrometers and the range of LET is between 4 to 26 KeV/µm. Auger electrons induce multiple lethal DNA DSBs in cancer cells, particularly if these electrons are released in close proximity to nuclear DNA. Auger electrons are emitted by radionuclides that decay by electron capture (EC) and/or internal conversion (IC). In these processes the proton-rich nucleus of atom absorbs an inner atomic electron, usually from the K or L electron shell. The vacancies created are filled by electrons decaying from higher shells creating subsequent vacancies that move progressively towards the outer shells. These transitions are ultimately accompanied by the emission of either a characteristic X-ray or low-energy Auger or IC electron (191). The emission of on average 5 to 36 electrons following every EC and/or IC event transiently imparts a positive charge on the decaying atom and deposits highly localized energy surrounding the site of decay. The lethal DNA damaging effects of Auger electrons is dependent on the position of radionuclide decay relative to the DNA (200). In order to exploit the high LET of these electrons and achieve a high RBE, decay of Auger electron emitters should be in close proximity to nuclear DNA (201-204). Dosimetric estimates showed that the Auger electron-emitter, 125I deposits the highest radiation absorbed dose in the immediate vicinity of the decay site (10⁹ cGy/decaying atom) with increasing distance up to a few nanometers from the DNA.
duplex (201, 202, 205, 206). When Auger electrons are emitted near but not within the DNA, DSBs are caused indirectly by hydroxyl radical (•OH)-mediated ionizations, however electrons still efficiently generates single-strand breaks (SSB) (207, 208). Some studies have exploited nuclear translocation sequence (NLS) peptides for mediating nuclear transport of monoclonal antibodies (mAbs) labeled with the Auger electron-emitter, $^{111}$In following receptor-mediated internalization by cancer cells (209-211). Dosimetric estimates revealed that the mean lethal dose of $^{125}$I conjugated to iododeoxyuridine (IUdR) which is incorporated directly into DNA is 7-fold lower than X-radiation for killing mammalian cells (206). In contrast, the cytotoxic effects of the Auger electron-emitters, $^{51}$Cr, $^{67}$Ga, $^{75}$Se, $^{125}$I, or $^{201}$Tl bound to the cell membrane (204, 212) or located inside (201-204) or outside the cell were no different from low LET $\gamma$-radiation [i.e. RBE < 2].

Pouget et al. studied the cytotoxicity of $^{125}$I when it’s localized in the nucleus ($^{125}$I-labeled tat peptides), cytoplasm ($^{125}$I-labeled anti-EGFR mAb 225 or anti-HER2 trastuzumab mAbs) or membrane ($^{125}$I-labeled anti-carcinoembryonic antigen (CEA) 35A7 mAbs) of A431 epidermoid cancer cells or SK-OV-3 ovarian cancer cells. Due to the DNA damaging properties of the Auger electrons, nuclear $^{125}$I was the most cytotoxic agent, but membrane bound $^{125}$I proved more effective for killing cells than $^{125}$I deposited in the cytoplasm (213). According to the critical function of cell membrane in cell survival there seems to be no absolute need for internalization of mAbs for Auger electron RIT, although nuclear localization enhances the potency of Auger electrons. Ionizing radiation generates ceramide-enriched membrane microdomains which recruit pro-apoptosis factors or interfere with the binding of growth factors and survival proteins that trigger apoptosis (214-217).

Auger electron-emitters exhibit a localized “bystander effect” which promotes the apoptosis or sometimes paradoxically stimulates the growth of non-targeted tumour cells that are proximal to targeted cells (218-220). Co-inoculation of male Ncr nude mice with a mixture of unlabeled LS174T human colon cancer cells and $^{125}$I-labeled cells demonstrated significant tumour growth inhibition compared to inoculation of only unlabeled cells, whereas a mixture of unlabeled and $^{123}$I-labeled cells exhibited enhanced tumour growth compared to inoculation of only unlabeled cells (220). In a separate study, $^{123}$I demonstrated growth inhibitory effects on cancer cells exposed to medium from cells that bound $^{123}$I-labeled metaiodobenzylguanidine ($^{123}$I-
mIBG) and that decreased their clonogenic survival (CS) which is possibly mediated by release of cell toxins from irradiated cells (218). Due to the nanometer range of Auger electrons, they don’t have a cross-fire effect that can widely irradiate and affect non-targeted cells, however emission on the cell surface of higher energy Auger electrons with a longer range can have a more localized “cross-dose” effect in which targeted cells irradiate neighboring non-targeted cells. This may overcome heterogeneity in tumour localization of the radiolabeled mAbs and internalization by cancer cells (221). These cross-dose and bystander effects may contribute to the effectiveness of Auger electron RIT for treatment of cancer (221).

Several Auger electron-emitting radionuclides have been studied for radiolabeling mAbs (Table 1-7). There are several important factors that should be considered in selecting a radionuclide for Auger electron RIT including: i) the energy of the emitted electrons, ii) the ratio of penetrating forms of radiation (e.g. X-rays and γ-photons) compared to non-penetrating Auger electrons (p/e ratio), iii) the physical half-life (t1/2) of the radionuclide, and iv) the method for radiolabeling the mAb (191). What determines the toxicity of a radioisotope is the average electron energy not the total energy. If a radioisotope with high electron yield deposits a high energy this energy would be distributed among a large number of electrons and the energy portion of each electron would be decreased. For example ⁶⁷Ga with a total electron energy of 34.4 keV but low electron yield (5.0 electrons/decay; Table 1-5) results in a higher average electron energy compared to ¹¹¹In or ¹²⁵I, radionuclides which have total electron energies of 32.7 keV and 19.4 keV, but higher electron yield (14.9 and 15.1 electrons/decay, respectively) (191, 222). A dose limiting factor for using Auger electron emitters is the emission of penetrating forms of radiation. Although these forms of radiation especially γ-photons can be exploited for dosimetry and monitoring tumour response by SPECT imaging, these may contribute to normal organ toxicity, especially at the high doses of radioactivity required for effective RIT.
Table 1-7. Auger electron emitters for conjugation to mAb for RIT of cancer.

| Radionuclide | Production method | Electron yield per decay | Total electron energy (keV) | γ-emission (keV) and abundance | half-life (t1/2p) | Method for radiolabeling
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{125}$I</td>
<td>$^{124}$Xe(n,γ)$^{125}$Xe $\rightarrow$ $^{125}$I</td>
<td>25.8</td>
<td>19.4</td>
<td>36(7%)</td>
<td>57 days</td>
<td>Radioiodination</td>
</tr>
<tr>
<td>$^{123}$I</td>
<td>$^{124}$Xe(n,2n)$^{123}$Cs $\rightarrow$ $^{123}$Xe $\rightarrow$ $^{123}$I or $^{124}$Xe(p,pn)$^{123}$Xe $\rightarrow$ $^{123}$I</td>
<td>15.1</td>
<td>27.6</td>
<td>159(83%)</td>
<td>13.2 h</td>
<td>Radioiodination</td>
</tr>
<tr>
<td>$^{111}$In</td>
<td>$^{112}$Cd(p,2n)$^{111}$In</td>
<td>14.9*</td>
<td>32.7</td>
<td>171 (90%); 245 (94%)</td>
<td>2.8 days</td>
<td>Chelation by DTPA or DOTA</td>
</tr>
<tr>
<td>$^{67}$Ga</td>
<td>$^{68}$Zn(p,2n)$^{67}$Ga</td>
<td>5.0</td>
<td>34.4</td>
<td>93 (36%); 185 (20%); 300 (16%)</td>
<td>3.3 days</td>
<td>Chelation by DFO or DOTA</td>
</tr>
<tr>
<td>$^{99m}$Tc</td>
<td>$^{99}$Mo $\rightarrow$ $^{99m}$Tc</td>
<td>5.1</td>
<td>16.3</td>
<td>140 (98%)</td>
<td>6.0 h</td>
<td>Chelation by HYNIC or N$_2$S$_2$</td>
</tr>
<tr>
<td>$^{64}$Cu</td>
<td>$^{64}$Ni(p,n)$^{64}$Cu</td>
<td>4.0</td>
<td>18.2</td>
<td>$^{d}$511 (19%)</td>
<td>12.7 h</td>
<td>Chelation by DOTA or NOTA</td>
</tr>
</tbody>
</table>

*Some studies reported $^{111}$In emits 8 electrons per decay (223).
DFO: desferrioxamine
HYNIC: hydrazinonicotinamide
N$_2$S$_2$: diamide dimercaptide
Energy of the two annihilation γ-photons resulting from 64Cu positron emission
NOTA: 1,4,7-triazacyclononane-N,N',N''-triacetic acid.
1.6.1.3 β-particles

In neutron rich atoms, a neutron transforms into a proton by emitting an electron (β-particle). The mass of the emitted β-particles is 1/200 of the mass of a proton or neutron. β-particles are moderate to high energy electrons (0.50–2.30 MeV) that travel a relatively long range in tissues (0.05–12.00 mm) with low LET of 0.1–1.0 keV/µm resulting in low deposition of energy in tumour cells restricting the potency for killing these cells (224). The long path length of β-particles however can overcome the heterogeneous distribution of antibodies in tumour tissue through crossfire radiation but it also increases the bone marrow toxicity due to a cross-fire effect from circulating radiolabeled mAbs. Furthermore, most of the energy of the β-particles is deposited at the end of its track length, which may be outside the treatment volume of a single cell or cluster of tumour cells. In contrast Auger electrons have a maximum range of 100 µm that restrict their cross-fire to 10 cell diameters (225), and alpha particles deposit their energy over 5-10 cell diameters which would represent a small cluster of tumour cells. As such, β-emitters are not ideal for the treatment of small tumour burdens including micro-metastatic disease.
<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Production</th>
<th>$E_{\beta_{\text{max}}}$ (MeV)</th>
<th>$\gamma$-emission (keV) and abundance</th>
<th>Half-life</th>
<th>Method for radiolabeling</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{90}\text{Y}$</td>
<td>Sr (n, $\gamma$)$^{90}\text{Y}$</td>
<td>2.28</td>
<td>-</td>
<td>2.7 days</td>
<td>Chelation by DOTA</td>
</tr>
<tr>
<td>$^{131}\text{I}$</td>
<td>Th (n, $\gamma$)</td>
<td>0.81</td>
<td>364 (81%)</td>
<td>8 days</td>
<td>Radioiodination</td>
</tr>
<tr>
<td>$^{177}\text{Lu}$</td>
<td>$^{176}\text{Lu} (n, \gamma) ^{177}\text{Yb} \rightarrow ^{177}\text{Lu}$</td>
<td>0.5</td>
<td>208 (11%); 113 (6%)</td>
<td>6.7 days</td>
<td>Chelation by DOTA</td>
</tr>
<tr>
<td>$^{186}\text{Re}$</td>
<td>$^{185}\text{Re} (n, \gamma) ^{186}\text{Re}$, $^{186}\text{W}(p,n) ^{186}\text{Re}$</td>
<td>1.08</td>
<td>137 (9%); 122 (12%)</td>
<td>3.8 days</td>
<td>Chelation by N3S, HYNIC or $\text{N}_2\text{S}_2$</td>
</tr>
<tr>
<td>$^{188}\text{Re}$</td>
<td>$^{187}\text{W}(n,\gamma) ^{188}\text{W} \rightarrow ^{188}\text{Re}$</td>
<td>2.12</td>
<td>155 (15%)</td>
<td>17 hours</td>
<td>Chelation by N3S, HYNIC or $\text{N}_2\text{S}_2$</td>
</tr>
<tr>
<td>$^{67}\text{Cu}$</td>
<td>$^{68}\text{Zn}(p,2p) ^{67}\text{Cu}$</td>
<td>0.58</td>
<td>184.6 (48%); 93.3 (23%)</td>
<td>2.6 days</td>
<td>Chelation by DOTA or NOTA</td>
</tr>
</tbody>
</table>

N3S: triaminemonothiol
HYNIC: hydrazinonicotinamide
$\text{N}_2\text{S}_2$: diamide dimercaptide
$^{90}$Y and $^{131}$I are the most commonly used two radionuclides for RIT. $^{90}$Y delivers $\beta$ particles with 5 times more energy to the tumour than $^{131}$I (Table 1-8); it also has a more favourable half-life than $^{131}$I (2.5 days compared with 8 days). The longer path length of $^{90}$Y compared to $^{131}$I (5 mm vs 0.88 mm) may be advantageous for treatment of larger tumours or those with poor antibody penetration but it increases toxicity to the normal surrounding tissues. Non-tumour distribution of uncomplexed $^{90}$Y has been reported to mainly be to the bone and liver, that of $^{131}$I is to the thyroid. As opposed to $^{131}$I which also emits $\gamma$-rays, $^{90}$Y requires a surrogate $\gamma$-emitter (e.g. $^{111}$In) to estimate dosimetry by imaging (226). Once radiolabeled antibody is internalized into the cells, it undergoes lysosomal degradation. Radiolabeled amino acid (monoiodotyrosine) is rapidly transported out of the cell whereas $^{90}$Y-chelator catabolites are not recognized by amino acid transporter and remain trapped in the lysosome (227). Furthermore $\gamma$-radiation of $^{131}$I requires the shielding of hospital personnel and restrictions following patient discharge. Currently $^{90}$Y-ibritumomab tiuxetan (Zevalin) and previously, $^{131}$I-tositumomab (Bexxar) have been used for RIT of patients with indolent B-cell non-Hodgkin lymphomas (NHL) by targeting CD20. Both agents exhibit similar clinical outcomes but with unique clinical considerations and radiation precautions due to the reasons discussed above (228).

Recently there has been a lot of interest in using $^{177}$Lu for RIT. Table 1-9 shows a list of ongoing clinical trials found on ClinicalTrials.gov for treatment of cancer using $^{177}$Lu labeled radioimmunoconjugates (RICs). $^{177}$Lu is a therapeutic reactor-produced radiometal that emits $\beta$-particles [$E_\beta(max)=498$ keV (78.6%), $E_\beta(max)=385$ keV (9.1%) and $E_\beta(max)=176$ keV (12.2%)]. It is easily produced on a large scale and at low cost. The energy of $\beta$-particles emitted by $^{177}$Lu is interemEDIATE between that of $^{131}$I and $^{90}$Y offering high tumour toxicity and reduced toxicity to surrounding organs and to the bone marrow. Low abundance $\gamma$-ray [$E_\gamma=113$ keV (6.4%) and $E_\gamma=208$ keV (11%)] emitted by $^{177}$Lu allows for SPECT imaging without the complication associated with high abundance $\gamma$-ray emitted by $^{131}$I [$E_\gamma=364$ keV (81%)] with regard to the shielding and patient discharge. These characteristics make $^{177}$Lu an attractive option for therapy of cancers. However, the low abundance of the gamma emissions from $^{177}$Lu limit its usefulness for imaging, and often a more abundant gamma emitter such as $^{111}$In is used as a surrogate for imaging the biodistribution of the radiolabeled antibodies.
Table 1-9. Ongoing clinical trials for cancer treatment using $^{177}$Lu labeled RICs and radiopeptides.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Treatment</th>
<th>Mechanism</th>
<th>Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney Cancer, Head and Neck Cancer, Breast Cancer, Non-small Cell Lung Cancer (NSCLC), Colorectal Cancer, Pancreatic Cancer, Ovarian Cancer, Esophageal Cancer, Gliomas</td>
<td>$^{177}$Lu-J591</td>
<td>J591: Anti-PSMA mAb</td>
<td>Not applicable</td>
</tr>
</tbody>
</table>
| Prostate Cancer | $^{177}$Lu-J591, Ketoconazole, Hydrocortisone, $^{111}$In-J591 | - Ketoconazole: inhibition of the activity of enzymes necessary for the conversion of cholesterol to steroid hormones such as testosterone and cortisol (229, 230)  
- Hydrocortisone: to compensate for cortisol insufficiency | Phase II |
| Prostate Cancer | $^{177}$Lu-J591, Docetaxel, Prednisone | - Docetaxel: Inhibition of mitotic cell division by preventing physiological microtubule depolymerisation/disassembly in the absence of GTP (231, 232)  
- Prednisone: Downregulation of glucocorticoids synthesis | Phase I |
| Small Cell Lung Cancer (NSCLC), Non-small Cell Lung Cancer (NSCLC) | TF2 mAb pre-targeting $^{177}$Lu-IMP-288, $^{111}$In-IMP-288 | - TF2: anti-CEA/anti-HSG bispecific mAb  
- IMP-288 hapten HSG peptide | Phase I Phase II |
| Pancreatic Carcinoma | MVT-1075 with blocking dose of MVT-5873 | - 5B1: anti CA19-9 mAb  
- MVT-5873: Unlabelled 5B1 mAb  
- MVT-1075: $^{177}$Lu- MVT-5873 | Phase I |
| Small Cell Lung Cancer | Nivolumab $^{177}$Lu-DOTA-TATE | - Nivolumab: Anti PD-1 mAb  
- DOTA-TATE: Targeting somatostatin receptor | Phase I Phase II |
| Metastatic Renal Cell Carcinoma | $^{111}$In-DOTA-cG250, $^{177}$Lu-DOTA-cG250 | cG250: Anti G250/CAIX/MN mAb | Phase I Phase II |
| Diffuse Large B-cell Lymphoma | Betalutin ($^{177}$Lu-lilotomab satetraxetan) | Betalutin: Anti-CD37 mAb | Phase I |

PSMA: prostate specific membrane antigen  
HSG: histamine-succinyl-glycine  
PD-1: Programmed cell death-1  
CEA: carcinoembryonic antigen  
Dotatate: DOTA0-Tyr3-Octreotate
Recently a randomized Phase 3 clinical trial of $^{177}$Lu-DOTA0-Tyr3-Octreotate (177Lu-DOTATATE; Figure 1-9) in 229 patients with inoperable, progressive, somatostatin receptor positive midgut carcinoid tumours (NETTER-1) showed markedly longer progression-free survival and a significantly higher response rate than high-dose octreotide long-acting repeatable (LAR) among patients with advanced midgut neuroendocrine tumours (233). $^{177}$Lu has application for radiolabeling peptides (e.g. $^{177}$Lu-DOTATATE) as well as mAbs for radiotherapeutic purposes (Table 1-9).
Figure 1-9. The chemical structure of $^{177}$Lu-DOTATATE
In summary, due to their potentially greater potency and lower non-specific toxicity to non-targeted normal tissues including the hematopoietic stem cells in the bone marrow, α-particle and Auger electron-emitting radionuclides are highly attractive for RIT of malignancies (Figure 1-10A). However, their short range makes these forms of radiation most suitable for eradicating single cells and small volume disease (<1 cm diameter) whereas the longer range of β-particles emitted by $^{177}$Lu or $^{90}$Y makes this form of radiation more feasible for treating larger tumour masses (>1 cm) (Figure 1-10B).
Figure 1-10. A) The cross-fire effect of the 2–10 mm range β-particles emitted by circulating radiolabeled mAbs perfusing the bone marrow (BM) decreases the viability of hematopoietic stem cells resulting in myelosuppression. In contrast, α-particles have a range of only 28–100 μm and Auger electrons have a range <0.5 μm which greatly reduces or eliminates the BM toxicity of radioimmunotherapy (RIT). B) Illustration of the track of α-particles, β-particles or Auger electrons emitted by radiolabeled mAbs targeted to cancer cells. The short track length of α-particles (28–100 μm) and Auger electrons (<0.5 μm) results in high linear energy transfer (LET) values of 50–230 keV/μm and 4 to 26 keV/μm, respectively. β-particles have a track length of 2–10 mm resulting in LET of 0.1–1.0 keV/μm. The high LET of α-particles (50–100 μm) and Auger electrons makes these forms of radiation more powerful for killing cancer cells than β-particles. Reprinted with permission from Aghevlian S et al. (191).
1.6.2 Clinical trials with RICs

Since the 1980s, clinical trials have tested numerous RICs for cancer but the field continues to develop with the introduction of new humanized and fully human antibodies and radionuclides (55). The results from clinical trials on the role of RIT as a neoadjuvant treatment to shrink a tumour to a resectable state is also controversial and conflicting (55, 234). ¹³¹I labeled anti-CEA mAb, Kab201, were the first RICs that were advanced to a phase I/II clinical trial for treatment of 18 patients with locally advanced or metastatic PnCa (235). The overall response rate was 6%. Several subsequent clinical trials have been performed with other RICs and will be discussed in this section.

1.6.2.1 ⁹⁰Y-clivatuzumab tetraxetan

Humanized clivatuzumab (hPAM4) mAb specifically targets the mucin glycoprotein MUC1 expressed on most PnCa. ⁹⁰Y-clivatuzumab tetraxetan (trade name hPAM4-Cide) is a radiopharmaceutical designed for the treatment of PnCa. In a Phase I clinical trial thirty-eight previously untreated patients received gemcitabine 200 mg/m² weekly for 4 weeks with ⁹⁰Y-hPAM4 given weekly in weeks 2, 3, and 4 (cycle 1), and the same cycle was repeated in 13 patients (cycles 2–4) (Figure 1-11).
Figure 1-11. The protocol schema of RIT indicates fractionated RIT; $^{111}$In-hPAM4/$^{90}$Y-hPAM4 indium-111 or yttrium-90 labeled humanized clivatuzumab tetraxetan; Gem: gemcitabine; PK, pharmacokinetics.
The maximum tolerated dose of $^{90}$Y-hPAM4 was determined to be 12.0 mCi/m$^2$ (444 MBq/m$^2$) weekly for 3 weeks for cycle 1, with $\leq$9.0 mCi/m$^2$ (333 MBq/m$^2$) weekly for 3 weeks for subsequent cycles. Six patients (16%) had partial responses and 16 patients (42%) had stabilization as their best response (58% disease control). The median overall survival was 7.7 months for all 38 patients, including 11.8 months for those who received repeated cycles (46% [6 of 13 patients] $\geq$1 year), with improved efficacy at the higher RIT doses (236).

In March 2016 the phase III PANCRT-1 trial in metastatic PnCa was terminated early due to lack of improvement of overall survival compared to gemcitabine alone (237). However, as mentioned, these patients had metastatic PnCa that had progressed on prior treatment regimens, and there remains the potential to impact patient outcome in PnCa if treatment was commenced earlier, especially in patients with surgically resectable tumours.

1.6.2.2 $^{131}$Iodine-Tenatumomab

Tenatumomab is a murine mAb developed by Sigma-tau directed towards Tenascin-C expressed on malignant pancreas, breast, colorectal, lung, ovarian or B and T cell Non-Hodgkin Limphoma tissue sections. The use of the $^{131}$I-labeled tenatumomab for anti-cancer RIT was tested in a phase I clinical trial, however due to negligible drug uptake into the tumour lesion this study was terminated (238).

1.6.2.3 $^{212}$Pb-TCMC-Trastuzumab

Alpha particle RIT using lead-212 ($^{212}$Pb), targeted to HER2 expressing cells by the trastuzumab antibody was studied, as a potential treatment for patients with metastatic HER-2 expressing tumours (e.g., ovarian, pancreatic, colon, gastric, endometrial, or breast). In this phase I trial 3 patients with HER-2 positive intraperitoneal cancers who had failed standard therapies received i.p injection of $^{212}$Pb-TCMC-trastuzumab after 4 mg/kg of trastuzumab. Pharmacokinetics and imaging after 0.2 mCi/m$^2$ (7.4 MBq/m$^2$) i.p. $^{212}$Pb-TCMC-trastuzumab in patients with HER-2-expressing malignancy showed minimal distribution outside the peritoneal cavity, $\leq$6% urinary excretion, and good tolerance (239). There is no information available on the effectiveness of treatment.
1.6.2.4 $^{90}$Y human mAb MN-14 (labetuzumab)

CEA has been recognized as an overexpressed cell surface antigen in many cancers, mainly in gastrointestinal tract malignancy. $^{90}$Y labeled humanized anti-CEA antibody MN14 was evaluated in phase I and II clinical trials to determine the safety of different dose levels in the treatment of PnCa ([https://clinicaltrials.gov/ct2/show/NCT00041639](https://clinicaltrials.gov/ct2/show/NCT00041639)). No information is available regarding the result of this trial.

1.6.2.5 $^{177}$Lu human mAb 5B1 (MVT-1075) in combination with a blocking dose of unlabeled 5B1 (MVT-5873)

MVT-1075, is a human antibody-based RICs developed by $^{177}$Lu labelling of 5B1 mAb directed towards CA19-9. Carbohydrate antigen 19-9, also called cancer antigen 19-9 or sialylated Lewis (a) antigen) is a tumour marker that used primarily in the management of PnCa. MVT-1075 is currently being evaluated in a phase I clinical study for the treatment of pancreatic, colon and lung cancer in combination a blocking dose of unlabeled 5B1 (MVT-5873) ([https://clinicaltrials.gov/ct2/show/NCT03118349](https://clinicaltrials.gov/ct2/show/NCT03118349)).

1.7 Increasing the specific activity (SA)

Increasing the specific activity of RICs may improve their potency for killing cancer cells and their effectiveness of cancer treatment. Specific activity (SA) is the amount of radioactivity carried by a RIC. Chelator complexes with radiometal in a 1:1 reaction, therefore the more chelator present on an antibody the more radioactivity will be delivered to a cancer cell. Studies have shown that amplification of radioactivity on a molecule of antibody using metal chelating polymers (MCPs) or dendrimers can not only eradicate cancer cells more efficiently (240, 241) but also overcome resistance to radiation in some cases (211). Furthermore, conjugation of antibodies to MCPs or dendrimers provides reproducible labelling without interfering trace metals. The concentration of trace metal contaminants increases with aging (decay) of the radioisotope stock solution. Competition of contaminants with the radioisotope to complex the chelator, can significantly reduce the labeling yield, while the chance of incorporating radiometal is higher with a large number of chelators on the antibody.
1.7.1 Dendrimer

Dendrimers are homogeneous nano-sized, radially symmetric molecules. Their monodisperse hyperbranched structure has a symmetric core, an inner shell, and an outer shell allowing for versatile functionalization (242) (Figure 1-12). Dendritic macromolecules tend to linearly increase in diameter and adopt a more globular shape with increasing dendrimer generation.

Figure 1-12. Three main parts of a dendrimer: the core, end-groups, and subunits linking the two molecules.
The biological properties of dendrimers such as polyvalency, self-assembling, electrostatic interactions, chemical stability, low cytotoxicity, and solubility renders them a promising candidate in cancer therapy and imaging fields. Polyvalency provides for versatile functionalization. The end-groups reaching the outer periphery of dendrimers can be conjugated to chelating agents, targeting moieties, contrast agents for magnetic resonance imaging (MRI) and drugs (243) (Figure 1-13).

Figure 1-13. Dendrimer with multiple surface functional groups (e.g. drugs, radioisotope, and antibodies) can be directed to cancer cells by tumour-targeting entities such as antibodies specific for tumour-associated antigens.
Dendrimers could be substituted with multiple metal chelators for radiolabeling to a high SA, and conjugated to a mAb for tumour targeting (244, 245). The presence of large numbers of atoms on a dendrimer molecule enables delivery of sufficient numbers of complexed metals as MRI contrast agents, diagnostic or therapeutic radioisotopes to cancer cells. For neutron capture therapy (NCT) of gliomas, Wu et al. developed boron-10 (\(^{10}\)B) radiolabeled dendrimers conjugated to anti-EGFR mAb cetuximab (IMC-C225) for targeted delivery of sufficient \(^{10}\)B to brain tumours in rats (1100 atoms of \(^{10}\)B per molecule of dendrimer) (244). Polyamidoamine dendrimers were modified with either 10 DOTA or 10 DTPA and were coupled to mAb 2E4. Both the DTPA- and DOTA-dendrimer-antibody constructs were then labeled with \(^{90}\)Y, \(^{111}\)In, \(^{212}\)Bi or stable Gd (III). Wu et al reported that conjugation of dendrimer to mAb 2E4 increased the labeling of \(^{90}\)Y to about a 47 fold higher SA compared to \(^{90}\)Y-labeled mAb 2E4 derivatized with one chelator per mAb 2E4 (1.74 MBq/μg vs. 0.037 MBq/μg) (246). In another study dendrimers were conjugated to 43 derivatives of DTPA, 2-(p-isothiocyanatobenzyl)-6-methyl-diethylene triamine penta-acetic acid (1B4M) for radiolabeling with \(^{111}\)In. Kobayashi et al. demonstrated that \(^{111}\)In-G4-1B4M dendrimer conjugated to a murine mAb IgG1 OST7 (470 MBq/μg) was labeled to a 54-fold higher SA than \(^{111}\)In-1B4M-OST7 (8.7 MBq/μg) (247). In our research group, dendrimers were reacted with 30 DTPA units then conjugated through a thiol to maleimide-derivatized trastuzumab for labeling with \(^{111}\)In (128). DTPA-denrimer-trastuzumab was further derivatized with NLS peptides for nuclear importation. The substitution level of dendrimer was 1 or 2 dendrimers per trastuzumab. \(^{111}\)In labeling of DTPA-dendrimer-trastuzumab yielded a 47 fold higher SA than \(^{111}\)In-DTPA-NLS-trastuzumab (23.6 MBq/μg vs. 0.5 MBq/μg). Clonogenic assays showed that high SA \(^{111}\)In-DTPA-denrimer RICs (5.5 MBq/μg) were 2-4 fold and 9 fold more cytotoxic to HER2 overexpressing SKBR-3 cells (1.3×10^6 receptors/cell) and low-HER2 density MDA-MB-231 cells (5×10^4 receptors/cell) than low SA \(^{111}\)In-DTPA-NLS-trastuzumab (0.5 MBq/μg) (241). High SA of \(^{111}\)In-dendrimer-trastuzumab overcomes the previously shown resistance of MDA-MB-231 cells to \(^{111}\)In-DTPA-NLS-trastuzumab (211).

These studies show the great potential of chelate-functionalized dendrimers to greatly increase the SA of mAb when labeled with radiometals compared to its direct conjugation to metal chelators. High SA labeled mAbs can be more cytotoxic to cancer cells with high and low expression of a particular target receptor such as HER2 (240).
1.7.2 Metal Chelating Polymer (MCP)

Polymers like dendrimers can be modified with metal chelators to serve the purpose of increasing the specific activity for radiometal labeling of antibodies and thus, amplification of radioactivity delivered to cancer or other diseased cells. Slinkin et al. conjugated Fab fragments of RD110 mAb raised against cardiac myosin with Polylysine (PL) chelating polymers carrying 40 DTPA chelators for labeling $^{111}$In (248). Unlike normal myocardial cells, necrotic cardiomyocytes cannot prevent binding of anti-myosin antibodies to intracellular myosin allowing for imaging of necrotic cardiomyocytes by radiolabeled antmyosin mAbs. PL-DTPA-Fab was labeled with $^{111}$In to a 50 fold higher SA (3.7 MBq/μg) than DTPA-Fab derivatized with DTPA only. In vivo studies in New Zealand rabbits with experimental myocardium infarction with $^{111}$In-PL-DTPA-Fab showed good accumulation of the labeled conjugate in infracted areas with a target to normal ratio as high as 25:1 (248).

More recently, Dr. Mitch Winnik’s research group (Department of Chemistry, University of Toronto) has been synthesizing MCPs for simultaneous detection of multiple cell specific biomarkers on individual cells by high throughout Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (249-251). In addition to carrying DOTA chelators for complexing lanthanides and increasing the sensitivity of the method, this water soluble MCP also has a functional maleimide end group allowing covalent binding to a mAb. Selective reduction of the disulfide bonds in the hinge region of the mAb creates thiols which reacts with the maleimide end group (132). ICP-MS studies showed that mAbs conjugated to lanthanides-labeled MCPs determined the relative expression of CD33, CD34, CD38, CD45 and CD54 on two leukemic cells lines (KG1a; THP-1) (249). Besides, multiple mAbs conjugated to MCPs but labeled with different lanthanides could be used in a single assay without signal interference. Collaboration with Dr. Winnik’s research group led to the development and synthesis of analogous MCPs to complex radiometals studied in this thesis (Figure 1-14). Previously, another type of MCP with polyglutamide (PGlu) backbone and 24-29 DTPA chelators (PGlu (DTPA)_{24-29}) was synthesised by Dr. Winnik’s research group. Oxidation of glycans in the Fc domain of antibodies with sodium-meta periodate (NaIO$_4$) forms aldehyde groups which will further react with the hydrazide end group on the MCP to form a hydrazone bond between the antibody and MCPs (240, 252). This method is called site-directed coupling and preserves the antigen binding sites.
from any chemical modification. Our group showed that the maximum achievable SA for $^{111}$In labeling of trastuzumab-MCP was 90-fold higher compared to trastuzumab directly modified with two DTPA, and this enhanced the cytotoxic potency \textit{in vitro} against HER2-positive breast cancer cells through the emission of Auger electrons by $^{111}$In (240).

![Figure 1-14. Metal chelating polymer consists of a backbone harbouring multiple pendant chelators for radiometal labeling to high specific activity as well as a functional end group for conjugation to a mAb.](image)
Dr. Winnik’s research group also synthesised an array of MCPs with varied backbones including polyacrylamide (PAm), polyaspartamide (PAsp), or polyglutamide (PGlu) and multiple DTPA units for labeling to the mAb to high SA with $^{111}$In. The shared feature of these MCPs was a biotin end group for reaction to streptavidin derivatized trastuzumab Fab fragments (SAv-Fab) (253-255). Bi-PAm(DTPA)$_{40}$ had a net negative charge while Bi-PAsp(DTPA)$_{33}$ and Bi-PGlu(DTPA)$_{28}$ were zwitterionic with a net neutral charge. Biodistribution studies of the MCPs conjugated to trastuzumab Fab fragments in Balb/c mice showed that zwitterionic MCPs have the most desirable properties with lower liver and spleen uptake (255). This study also showed that saturation of DTPA chelators on Bi-PAm(DTPA)$_{40}$ with stable $^{115}$In in order to reduce the negative charge on the polymers also reduced the spleen and liver uptake. Polyethylene glycol (PEG) modification may also improve the biodistribution profile of MCP-conjugated mAbs since PEG can shield against opsonization by plasma proteins which promotes uptake by macrophages and increases liver and spleen accumulation (256). Therefore a new generation of metal chelating polymer (MCP) reagents were developed in this thesis that carry multiple polyethylene glycol (PEG) pendant groups to provide stealth to MCP-based radioimmunoconjugates (RICs). A hydrazinonicotinamide (HyNic) group was installed on the initiating end of this new generation of MCP (HyNic-MCP). The conjugation of this functional end group and an aromatic aldehyde introduced on ε-NH$_2$ groups of lysines in antibodies forms a bis-aromatic hydrazone bond with a measurable absorbance at 350 nm (257). Boyle et al. reported that F(ab’)$_2$ of the anti-EGFR mAb, panitumumab conjugated to an MCP with a PGlu backbone incorporating 2-8 PEG (2 kDa) chains and 13-15 DOTA for complexing $^{64}$Cu, exhibited low liver and kidney uptake and good tumour uptake [5-8 percent injected dose/g (%ID/g)] in human PnCa xenografts in NOD-scid mice, allowing tumour imaging by PET (258).

In my thesis research project, a MCP with a PGlu backbone incorporating 10 PEG moieties and presenting on average 13 DOTA that complex $^{111}$In or $^{177}$Lu conjugated to panitumumab via hydrazine nicotinamide (HyNic) chemistry was studied in comparison to DOTA conjugated panitumumab. More details are provided in Chapters 2 and 3. The overall aim of the research was to develop $^{111}$In or $^{177}$Lu labeled MCP-panitumumab theranostics, and compare their in vitro and in vivo properties with their DOTA-conjugated panitumumab counterparts under the same conditions. The feasibility of providing reproducible labeling and high SA, may improve the therapeutic outcome with these MCP radioimmunoconjugates.
1.8 Hypothesis

The hypothesis of this thesis is that anti EGFR mAb panitumumab labeled with the Auger electron emitter, $^{111}$In or the $\beta$-particle emitter, $^{177}$Lu will be useful agents for molecular imaging and RIT of PnCa xenografts in mice. Modification of panitumumab with HyNIC-MCP that harbours 13 pendant DOTA chelators and 10 PEG moieties to reduce liver and spleen uptake could allow for dual labeling with $^{111}$In and $^{177}$Lu, more reproducible radiolabeling and higher SA and potentially higher potency for killing cancer cells.

1.9 Objective and Aims

The overall objective of this research was to develop novel RICs for molecular imaging and RIT of PnCa. The specific aims were:

I) To synthesis and characterize $^{111}$In- and/or $^{177}$Lu-labeled panitumumab-MCP and panitumumab-DOTA conjugates in terms of specific activity (SA), stability, EGFR binding affinity, biodistribution profile and to evaluate the imaging capability of $^{111}$In-labeled panitumumab conjugates to visualize s.c. PANC-1 human PnCa xenografts in NOD/SCID mice using microSPECT/CT imaging.

II) To evaluate in vitro toxicity and cellular dosimetry of panitumumab-MCP-$^{177}$Lu toward PANC-1 cells as well as their normal tissue toxicity, tumour growth inhibitory effects and absorbed doses in tumour and normal tissues in NRG mice engrafted s.c. with PANC-1 xenografts.

III) To evaluate and compare in vitro toxicity and cellular dosimetry of panitumumab-DOTA-$^{177}$Lu, panitumumab-DOTA-$^{111}$In and panitumumab-MCP-$^{111}$In towards PANC-1 cells as well as their normal tissue toxicity, tumour growth inhibitory effects and absorbed doses in tumour and normal tissues in NRG mice and NOD/SCID mice engrafted s.c. with PANC-1 xenografts.

The methodology, results and discussion of the research addressing these three aims are described in chapter 2, 3 and 4 of the thesis. The conclusion of the thesis and a discussion of the future research directions are provided in chapter 5.
Chapter 2: Panitumumab Modified with Metal-Chelating Polymers (MCP) Complexed to $^{111}$In and $^{177}$Lu – An EGFR-Targeted Theranostic for Pancreatic Cancer

All experiments were designed and carried out by Sadaf Aghevlian. MicroSPECT/CT was performed with technical assistance from Deborah A Scollard and Teesha Kamal. The synthesis and characterization of metal chelating polymer were performed by Dr. Yijie Lu at the Department of Chemistry at the University of Toronto.
2. Abstract

A metal-chelating polymer (MCP) with a polyglutamide (PGlu) backbone presenting on average 13 DOTA (tetraazacyclododecane-1,4,7,10-tetraacetic acid) chelators for complexing $^{111}$In or $^{177}$Lu and 10 polyethylene glycol (PEG) chains to minimize liver and spleen uptake was conjugated to antiepidermal growth factor receptor (EGFR) monoclonal antibody (mAb), panitumumab. Because panitumumab-MCP may be dual-labeled with $^{111}$In and $^{177}$Lu for SPECT, or radioimmunotherapy (RIT) exploiting the Auger electrons or β-particle emissions, respectively, we propose that panitumumab-MCP could be a useful theranostic agent for EGFR-positive PnCa. Bioconjugation was achieved by reaction of a hydrazine nicotinamide (HyNIC) group on the MCP with an aryl aromatic aldehyde introduced into panitumumab by reaction with succinimidyl-4-formylbenzamide (S-4FB). The conjugation reaction was monitored by measurement of the chromophoric bis-aryl hydrazone bond formed ($\varepsilon_{350\text{ nm}} = 24\,500\,\text{M}^{-1}\text{cm}^{-1}$) to achieve two MCPs/panitumumab. Labeling of panitumumab-MCP with $^{111}$In or $^{177}$Lu demonstrated that masses as small as 0.1 μg were labeled to >90% labeling efficiency (L.E.) and a specific activity (SA) of >70 MBq/μg. Panitumumab-DOTA incorporating two DOTA per mAb was labeled with $^{111}$In or $^{177}$Lu to a maximum SA of 65 MBq/μg and 46 MBq/μg, respectively. Panitumumab-MCP-$^{177}$Lu exhibited saturable binding to EGFR-overexpressing MDA-MB-468 human breast cancer cells. The Kd for binding of panitumumab-MCP-$^{177}$Lu to EGFR (2.2 ± 0.6 nmol/L) was not significantly different than panitumumab-DOTA-$^{177}$Lu (1.0 ± 0.4 nmol/L). $^{111}$In and $^{177}$Lu were stably complexed to panitumumab-MCP. Panitumumab-MCP-$^{111}$In exhibited similar whole body retention (55–60%) as panitumumab-DOTA-$^{111}$In in NOD-scid mice up to 72 h postinjection (p.i.) and equivalent excretion of radioactivity into the urine and feces. The uptake of panitumumab-MCP-$^{111}$In in most normal tissues in NOD-scid mice with EGFR-positive PANC-1 human pancreatic cancer (PnCa) xenografts at 72 h p.i. was not significantly different than panitumumab-DOTA-$^{111}$In, except for the liver which was 3-fold greater for panitumumab-MCP-$^{111}$In. Tumour uptake of panitumumab-MCP-$^{111}$In (6.9 ± 1.3% ID/g) was not significantly different than panitumumab-DOTA-$^{111}$In (6.6 ± 3.3%ID/g). Tumour uptake of panitumumab-MCP-$^{111}$In and panitumumab-DOTA-$^{111}$In were reduced by preadministration of excess panitumumab, suggesting EGFR-mediated uptake. Tumour uptake of nonspecific IgG-MCP (5.4 ± 0.3%ID/g) was unexpectedly similar to panitumumab-MCP-$^{111}$In.
An increased hydrodynamic radius of IgG when conjugated to an MCP may encourage tumour uptake via the enhanced permeability and retention (EPR) effect. Tumour uptake of panitumumab-DOTA-\textsuperscript{111}In was 3.5-fold significantly higher than IgG-DOTA-\textsuperscript{111}In. PANC-1 tumours were imaged by microSPECT/CT at 72 h p.i. of panitumumab-MCP-\textsuperscript{111}In or panitumumab-DOTA-\textsuperscript{111}In. Tumours were not visualized with preadministration of excess panitumumab to block EGFR, or with nonspecific IgG radioimmunoconjugates. We conclude that linking panitumumab to an MCP enabled higher SA labeling with \textsuperscript{111}In and \textsuperscript{177}Lu than DOTA-conjugated panitumumab, with preserved EGFR binding in vitro and comparable tumour localization in vivo in mice with s.c. PANC-1 human PnCa xenografts. Normal tissue distribution was similar except for the liver which was higher for the polymer radioimmunoconjugates.
Graphical abstract
2.1 Introduction

Radioimmunotherapy (RIT) employs monoclonal antibodies (mAbs) recognizing tumour-associated receptors/antigens complexed to radionuclides that emit α-particles, β-particles or Auger electrons for systemic radiation treatment of cancer (191, 259). RIT has been proven in principle in a broad diversity of cancers in mouse tumour xenograft models and has been effective for treatment of non-Hodgkin’s B-cell lymphoma in cancer patients (260). RIT of solid tumours has been more challenging because of limited delivery and penetration of radiolabeled mAbs into these tumours combined with dose-limiting hematopoietic system toxicity (261). The barriers to delivery of radiolabeled mAbs to solid tumours include poor extravasation, high tumour interstitial pressure (262), aberrant tumour vascularization (263), stromal barriers (264), and a binding site barrier, which restricts deep tumour penetration due to binding to tumour cells near blood vessels (265). Low and heterogeneous receptor/antigen expression have also been reported to diminish the effectiveness of antibody-based therapies (266) and may play a role in decreasing the effectiveness of RIT.

One strategy to overcome these delivery barriers may be to enhance the amount of radioactivity targeted to tumour cells per receptor/antigen binding event through increasing the proportion of antibody molecules carrying a radionuclide (i.e., specific radioactivity (SA)). Radiolabeling is most commonly performed by conjugation of chelators to the mAbs such as DTPA (diethylenetriaminepentaacetic acid) or DOTA (tetraazacyclododecane-1,4,7,10-tetraacetic acid) that form a complex with radiometals, but to minimize the effect of chelator substitution on binding affinity, usually only one to two DTPA or DOTA are conjugated per mAb molecule (267). This low chelator substitution level limits the SA that is achievable, such that there may be as few as 1 in 50 antibody molecules that are radiolabeled (240). To address this challenge, we previously reported the synthesis of a metal-chelating polymer (MCP) composed of a polyglutamide (PGlu) backbone presenting 24 or 29 DTPA per MCP for complexing $^{111}$In (268). This MCP was conjugated to the Fc-domain of antihuman epidermal growth factor receptor-2 (HER2) mAb, trastuzumab (Herceptin, Roche) with good preservation of HER2 binding (Kd = 1 × 10^{-8} mols/L) (240). The SA achieved for labeling trastuzumab-MCP with $^{111}$In was increased up to 90-fold compared with trastuzumab directly modified with two DTPA, and this increased
the cytotoxic potency in vitro against HER2-positive breast cancer cells through the emission of Auger electrons by $^{111}$In.

Nonetheless, an important consideration in the application of MCPs for high SA radiolabeling of mAbs is their effect on tumour and normal tissue uptake. We previously examined the effect of conjugation of MCPs with different backbones [polyacrylamide (PAm), polyaspartamide (PAsp), or polyglutamide (PGlu)] that presented 28–40 DTPA for complexing $^{111}$In on the tumour and normal tissue distribution of trastuzumab Fab in mice with s.c. HER2-positive SK-OV-3 human ovarian cancer xenografts (269). Liver uptake was 5–13-fold higher for Fab conjugated to the PAm MCP compared with a MCP with a PAsp or PGlu backbone. However, polymer charge was also important, because saturation of uncomplexed DTPA on PAm MCP with stable indium ($^{115}$In) to eliminate residual anionic charges, decreased liver uptake by 1.5-fold. The highest tumour uptake was observed with PAsp MCP-conjugated Fab. We then compared a PAm MCP with 36 DTPA linked via a diethylenetriamine (DET) group to an MCP with 40 DTPA linked via an ethylenediamine (EDA) group (270). The DET-functionalized polymer was zwitterionic when DTPA was complexed to $^{111}$In or saturated with stable indium, whereas the EDA polymer was anionic. In mice with s.c. SK-OV-3 tumours, zwitterionic polymer conjugates exhibited decreased liver and kidney uptake, enhanced retention in the blood, and improved tumour uptake compared with those conjugated to the anionic EDA MCP. Polyethylene glycol (PEG) modification may also improve the biodistribution profile of MCP-conjugated mAbs since PEG can shield against opsonization by plasma proteins which promotes uptake by macrophages and increases liver and spleen uptake (256). We reported that F(ab')2 of the antiepidermal growth factor receptor (EGFR) mAb, panitumumab (Vectibix, Amgen) conjugated to an MCP with a PGlu backbone incorporating 2–8 PEG (2 kDa) chains and 13–15 DOTA for complexing $^{64}$Cu, exhibited low liver and kidney uptake and relatively good tumour uptake [5–8% injected dose/g (%ID/g)] in human pancreatic cancer (PnCa) xenografts in NOD-scid mice, allowing tumour imaging by positron-emission tomography (PET) (271).

In the current study, we describe for the first time a MCP with a PGlu backbone that incorporates on average 10 PEG chains and 13 DOTA to complex $^{111}$In or $^{177}$Lu conjugated to panitumumab via hydrazine nicotinamide (HyNic) chemistry (272). $^{111}$In [t1/2 = 2.8 days] emits 15 low energy (<25 keV) Auger electrons that have a subcellular range ideal for killing single tumour cells or
small clusters of tumour cells (e.g., micrometastases). In addition, $^{111}$In emits two high abundance $\gamma$-photons [$E_\gamma = 171$ keV (90%) and $E_\gamma = 245$ keV (94%)] that enable single photon emission computed tomography (SPECT) imaging. $^{177}$Lu [$t_{1/2} = 6.7$ days] emits moderate energy $\beta$-particles [$E_{\beta(\text{max})} = 498$ keV (78.6%), $E_{\beta(\text{max})} = 385$ keV (9.1%) and $E_{\beta(\text{max})} = 176$ keV (12.2%)] that have a longer 2 mm range suitable for RIT of larger tumours. $^{177}$Lu also emits two low abundance $\gamma$-photons [$E_\gamma = 113$ keV (6.4%) and $E_\gamma = 208$ keV (11%)] that can be imaged by SPECT. Since the MCP provides multiple DOTA chelators for complexing $^{111}$In and $^{177}$Lu, this provides an opportunity for dual-labeling of panitumumab to allow imaging as well as RIT of different sized tumour deposits (“theranostic” approach). The higher abundance $\gamma$-photons emitted by $^{111}$In would also increase the sensitivity of SPECT compared with imaging using only the low abundance $\gamma$-photons emitted by $^{177}$Lu. Our aim in this study was to conjugate panitumumab to these MCPs and compare the properties of the immunoconjugates to panitumumab directly modified with DOTA. These properties included complexation of $^{111}$In and $^{177}$Lu to high SA, EGFR immunoreactivity, stability and tumour and normal tissue distribution including SPECT imaging in NOD-scid mice with s.c. PANC-1 human PnCa xenografts. Panitumumab binds with high affinity to the EGFR ($K_d = 5 \times 10^{-11}$ M) (273) which is overexpressed on >90% of PnCa tumours (274).

2.2 Materials and Methods

2.2.1 Cell Culture and Tumour Xenografts

PANC-1 human pancreatic adenocarcinoma cells ($4 \times 10^5$ EGFR/cell) (143) and MDA-MB-468 human breast cancer cells ($1.3 \times 10^6$ EGFR/cell) (275) were obtained from the American Type Culture Collection (ATCC). Both cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with high glucose supplemented with 1% penicillin and streptomycin and 10% fetal bovine serum (Gibco-Invitrogen). Tumour xenografts (6–7 mm diameter) were established in female nonobese diabetic severe combined immunodeficiency (NOD-scid) mice (Charles River) by subcutaneous (s.c.) inoculation of $4 \times 10^6$ PANC-1 cells in serum free DMEM (100 μL) into the left abdomen. All animal studies were conducted in compliance with Canadian Council on Animal Care (CCAC) guidelines and were carried out under a protocol approved by the Animal Care Committee at the University Health Network (AUP 2843.3).
2.2.2 Radioimmunoconjugates (RICs)

Hydrazino nicotinamide (HyNic) end-capped metal-chelating polymers (MCP; Figure 2-1) were synthesized and characterized as reported by Lu et al (271) Panitumumab (Vectibix; Amgen) was reacted with succinimidyl-4-formylbenzamide (S-4FB; synthesized in-house) for 2 h in phosphate-buffered saline (PBS), pH 8.0 to install aldehyde groups for reaction with HyNic-MCP. The number of S-4FB conjugated to panitumumab was quantified colorimetrically by reaction with 2-hydrazinopyridine (Sigma-Aldrich) which forms a chromophoric bis-aryl hydrazone that absorbs at 350 nm (ε350 nm = 24,500 M–1 cm–1). S-4FB-modified panitumumab was purified using an Amicon 30 kDa molecular weight cutoff (MWCO) spin filter and reacted with 5 equivalent of HyNic-MCP to form a bis-aryl hydrazone chromophore which absorbs at 354 nm (ε354 nm = 29,000 M–1 cm–1). The desired number of MCP per panitumumab was obtained by measuring the absorbance at 354 nm of the reaction mixture every few minutes until the absorbance corresponded on an average of two MCPs linked to panitumumab. Unconjugated polymers were removed from MCP-panitumumab by diluting first in 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer pH 7.4 to block any unreacted aldehydes and then in 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer pH 5.0 for subsequent radiolabeling, and passing through an Amicon spin filter (0.5 mL; 100 kDa MWCO; Millipore).
Figure 2-1. Chemical structure of the hydrazino nicotinamide metal chelating polymer (HyNic-MCP; Mr ~ 33 kDa); (B,C) Synthesis of panitumumab-HyNic-MCP; and (D) immunoconjugates are labeled with $^{111}\text{InCl}_3$ or $^{177}\text{LuCl}_3$ in 0.1 M HEPES Buffer, pH 5.5.

The polymer incorporates a polyglutamide backbone harboring on average 13 pendant DOTA groups as well as 10 PEG chains. The chemically reactive group, HyNic, is indicated by the blocked area.

Following derivatization of $\varepsilon$-NH$_2$ groups on lysine residues in panitumumab with S-4FB, the hydrazine group on the MCP interacts with installed aldehyde groups on panitumumab to form a bis-aryl hydrazone bond that absorbs at 354 nm. This reaction allows the measurement of the number of MCP conjugated to panitumumab as the reaction proceeds.
MCP-panitumumab was characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 4–20% Tris-HCl gel (Bio-Rad) stained with Coomassie blue. Molecular weight standards (10–260 kDa) were used to calibrate the gel and determine the molecular size of the immunoconjugates. For comparison, panitumumab was directly modified with DOTA by reaction with the N-hydroxysuccinimide ester (DOTA-NHS; Macrocyclics). Briefly, panitumumab (2 mg) was exchanged into metal-free NaHCO3 buffer pH 8.2 (100 μL), then reacted with 10 equivalents of DOTA-NHS for 2 h at 25 °C. The immunoconjugates were concentrated and purified using an Amicon spin filter (0.5 mL; 30 kDa MWCO; Millipore) in 0.1 M HEPES, pH 5.5 for subsequent radiolabeling. Similarly nonspecific human IgG (Sigma-Aldrich) was modified with DOTA or MCP for radiolabeling with 111In or 177Lu. DOTA conjugation efficiency was determined by trace-labeling a sample of the unpurified immunoconjugates (10 μL) with 111InCl₃ (Nordion) or 177LuCl₃ (PerkinElmer) at 42 °C for 3 h and measuring the proportion of 111In/177Lu-DOTA-panitumumab (Rf = 0.0) and unconjugated 111In/177Lu-DOTA (Rf = 1.0) by instant thin layer silica-gel chromatography (ITLC-SG; Pall) developed in 0.1 M sodium citrate, pH 5.5. The conjugation efficiency was multiplied by the 10-fold equivalent excess of DOTA in the reaction to calculate the number of DOTA/panitumumab.

The maximum specific activity (SA) achievable for 111In or 177Lu labeling of the immunoconjugates was studied by labeling decreasing masses of panitumumab-MCP or panitumumab-DOTA (100, 50, 10, 1, and 0.1 μg) with a constant amount (9.6 MBq) of 111InCl₃ or/and 177LuCl₃ and measuring the percent labeling efficiency (L.E.) by ITLC-SG. The feasibility of dual labeling of the immunoconjugates with 111In and 177Lu was studied by addition of 111InCl₃ (7.8 MBq) and 177LuCl₃ (5.7 MBq) to 1 μg of panitumumab-MCP and panitumumab-DOTA. Dual-isotope incorporation was measured by γ-counting with detection of the 177Lu X-ray peak at 60 keV and 111In sum peak at 428 keV. Corrections were applied to eliminate crossover from 111In to 177Lu and vice versa.

2.2.3 Measurement of Hydrodynamic Radius

To assess the effect of MCP conjugation on the molecular size of panitumumab, the hydrodynamic radius of the immunoconjugates was measured by size-exclusion chromatography (SEC, Viscotek) on tandem ViscoGEL G4000PWXL and G2500PWXL columns with
polymethacrylate beads maintained at 30 °C on a high performance liquid chromatography (HPLC) system equipped with a VE3210 UV/vis detector and VE3580 refractive index detector. The eluent used was 0.2 M KNO3, in 25 mM sodium phosphate buffer pH 8.5 containing 200 ppm of NaN3 and the flow rate was 1.0 mL/min delivered using a VE1122 Solvent Delivery System and VE7510 GPC Degasser (Viscotek). D-Glucose was used as an internal standard. A calibration curve was constructed by plotting the elution time of polyethylene glycols (PEG, 0.54–23.8 kDa, Polymer Laboratories) as well as protein standards (12.4–440 kDa, Sigma-Aldrich) vs their corresponding hydrodynamic radius (Rh). The scaling relationship (hydrodynamic radius vs molecular weight) of PEG and protein standards were obtained from the literature (276-280). On the basis of this relationship, the resulting hydrodynamic radius vs retention time calibration curve was plotted.

2.2.4 EGFR Immunoreactivity

The EGFR binding affinity of ¹⁷⁷Lu-labeled panitumumab-MCP was measured in saturation binding assays using MDA-MB-468 human breast cancer cells (1.3 × 10⁶ EGFR/cell) and compared to panitumumab-DOTA-¹⁷⁷Lu. Briefly, increasing concentrations (0 to 65 nmol/L) of ¹⁷⁷Lu-labeled immunoconjugates (50 μL) were incubated with 1 × 10⁶ MDA-MB-468 cells (100 μL) in 1.5 mL Eppendorf tubes at 4 °C for 3.5 h with gentle shaking. PBS (50 μL) was then added to the cell suspensions and the tubes were centrifuged on an Eppendorf microcentrifuge Model 5418 at 2,410 × g for 5 min to separate the unbound radioactivity in the supernatant from the cell pellet. The total cell-bound radioactivity (TB) in the pellets was measured in a γ-counter (PerkinElmer Wizard 3). The assay was repeated in the presence of a 50-fold molar excess of unlabeled panitumumab (0.003–3.2 μM) to measure nonspecific binding (NSB). Specific binding (SB) was calculated by subtracting NSB from TB and was plotted vs the concentration of ¹⁷⁷Lu-immunoconjugates added. The resulting curve was fitted by nonlinear regression to a one-site receptor-binding model by Prism Ver. 4.0 software (GraphPad). The dissociation constant (Kd) and maximum number of receptors per cell (Bmax) were calculated and compared for panitumumab-MCP-¹⁷⁷Lu and panitumumab-DOTA-¹⁷⁷Lu.
2.2.5 Stability of the RICs

The stability of $^{177}$Lu- or $^{111}$In-labeled panitumumab-MCP or panitumumab-DOTA RICs against transchelation to ethylenediaminetetraacetic acid (EDTA) was evaluated. The RICs (100 μg; 1 μg/μL) were incubated with a 500-fold molar excess of EDTA (3.3 mM; pH = 7.4; Sigma-Aldrich) for 1 h at room temperature. The proportion of $^{111}$In or $^{177}$Lu transchelated to EDTA or remaining bound to panitumumab-MCP or panitumumab-DOTA was measured by ITLC-SG (Rf = 1.0 and 0.0, respectively) and compared to RICs incubated in PBS, pH 7.0. In a separate experiment $^{177}$Lu- or $^{111}$In-labeled panitumumab-MCP or panitumumab-DOTA (0.6 MBq/μg) were incubated with human plasma (Sigma-Aldrich; P9523) in 0.5 mL Eppendorf tubes at a concentration of 0.037 MBq/μL for up to 7 days at 37 °C. Samples were analyzed in duplicate by size-exclusion HPLC (SE-HPLC) on a BioSep silica-based SEC-S2000 column (exclusion limit, 1000–300 000 kDa; Phenomenex) eluted with 100 mM NaH2PO4 buffer, pH 7.0, at a flow rate of 0.8 mL/min. The SE-HPLC system (PerkinElmer) was interfaced to a flow scintillation analyzer (FSA) radioactivity detector (PerkinElmer). Because HyNic-MCP interacts with the silica matrix of the HPLC column, the stability of $^{177}$Lu- or $^{111}$In-labeled panitumumab-MCP was alternatively studied by ultrafiltration through a Millipore centrifugal filter device (MWCO = 100 kDa; Amicon) and measuring the percentage of radioactivity filtered vs retained through the device in a γ-counter.

The stability of the RICs in vivo was assessed by measuring the whole body retention in mice and determining the proportion of radioactivity eliminated in the urine or feces. Groups of 4–5 female NOD-scid mice engrafted s.c. with PANC-1 cells were injected intravenously (i.v.) in the tail vein with 37 MBq (10 μg) of panitumumab-MCP-$^{111}$In or panitumumab-DOTA-$^{111}$In. The radioactivity retained in the whole body was measured in a radioisotope calibrator (Model CRC-12; Capintec) immediately after injection and at 1, 4, 24, 48, and 72 h post injection (p.i.). Mice were housed in metabolic cages and the cumulative percentage of the injected dose eliminated in the urine and feces was collected and measured in the dose calibrator at the corresponding time points.
2.2.6 Imaging and Biodistribution Studies

Imaging and biodistribution studies were performed in groups of 5 NOD-scid mice bearing s.c. PANC-1 tumours injected i.v. with 3.7 MBq (10 μg) of panitumumab-MCP-\(^{111}\)In or panitumumab-DOTA-\(^{111}\)In. To determine the specificity of tumour uptake, other groups of mice were injected with nonspecific human IgG labeled with \(^{111}\)In (IgG-DOTA-\(^{111}\)In or IgG-MCP-\(^{111}\)In). The specificity of tumour uptake was also assessed by EGFR blocking performed by i.v. administration of 1 mg of unlabeled panitumumab 1 day prior to injection of panitumumab-MCP-\(^{111}\)In or panitumumab-DOTA-\(^{111}\)In. At 72 h p.i. one or two representative mice were selected from each group and imaged on a NanoSPECT/CT tomograph (Bioscan) equipped with 4 NaI detectors and fitted with 1.4 mm multipinhole collimators [full width at half-maximum height (fwhm) = 1.2 mm]. For imaging, mice were anaesthetized by inhalation of 2% isoflurane in O\(_2\), and body temperature was maintained at 37 °C using a Minerve bed (Bioscan). A total of 24 projections were acquired in a 256 × 256 acquisition matrix with a minimum of 80,000 counts per projection. Images were reconstructed using an ordered-subset expectation maximization (OSEM) algorithm (9 iterations). Cone-beam CT images were acquired (180 projections, 1 s/projection, 45 kVp) and coregistration of micro-SPECT and CT images was performed using InvivoScope software. For some mice, CT was performed on an eXplore Locus Ultra Preclinical CT scanner (GE Healthcare) with routine acquisition parameters (80 kV, 50 mA-Voxel size of 154 × 154 × 154 μm) and coregistration of microSPECT and CT images performed using Siemens Inveon Research Workplace software. Mice were sacrificed by cervical dislocation under anesthesia, and the tumour and samples of normal tissues including blood were collected, weighed, and counted in a γ-counter. Radioactivity uptake was expressed as % ID/g.

2.3 Statistical Analysis

Results were expressed as mean ± SEM and tested for significance (P < 0.05) using a two-sided Student’s t test using Prism Ver. 4.0 software (GraphPad).
2.4 Results

2.4.1 Radioimmunoconjugates

Panitumumab was first reacted with S-4FB to install aldehyde groups for conjugation to HyNic-MCP (Figure 2-1). There were $4.1 \pm 0.7$ S-4FB groups on panitumumab based on the absorbance at 350 nm of the bis-aryl hydrazone chromophore produced after reaction with 2-hydrazinopyridine (281). S-4FB modified panitumumab was then reacted with an 5-fold excess of HyNic-MCP for a sufficient length of time (75 min) to achieve substitution of approximately two polymers per panitumumab molecule based on measurement of the bis-aryl hydrazone chromophore at 354 nm formed in the reaction (Figure 2-2A,B). Panitumumab-MCP was transferred to 0.1 M Tris pH 7.4 to block unreacted aldehydes and then to 0.1 M HEPES pH 5.0 to remove unconjugated MCP by ultrafiltration through an Amicon device. Reaction of panitumumab with a 10-fold excess of DOTA-NHS resulted in substitution of $2.2 \pm 0.5$ DOTA per antibody molecule. Analysis of panitumumab-MCP immunoconjugates by SDS-PAGE under reducing and non-reducing conditions showed a broad upward band shift from 150 kDa toward higher MW bands compared with panitumumab or panitumumab-DOTA indicating that there was some variability in the number of MCP conjugated to panitumumab. Two major bands were observed under reducing condition, at 50 and 25 kDa representing the heavy and light chains of panitumumab (Figure 2-2C).
Figure 2-2. (A) Reaction of panitumumab-S-4FB with HyNic-MCP was monitored by measuring the absorbance at 354 nm due to formation of the bis-aryl hydrazone chromophore. (B) The reaction was stopped at 75 min when the average number of MCP per panitumumab was 2, which corresponded to an absorbance of 0.79. (C) SDS-PAGE analysis of panitumumab and MCP- or DOTA-immunoconjugates on a 4–20% Tris-HCl gel stained with Coomassie Brilliant Blue under nonreducing and reducing (2-mercaptoethanol) conditions. Lanes: MW: MW markers (kDa), 2/5: panitumumab, 3/6: panitumumab-DOTA, 4/7: panitumumab-MCP. Under nonreducing condition, the upward band shift in lane 4 associated with an increase in molecular weight of panitumumab indicated the attachment of MCPs. Under reducing condition, there are two major bands at 25 kDa and 50 kDa for panitumumab, panitumumab-DOTA and panitumumab-MCP, which are the light and heavy chains of panitumumab, and an additional diffuse higher MW band for panitumumab-MCP.
Panitumumab-MCP and panitumumab-DOTA were labeled with $^{111}$In or $^{177}$Lu at high L.E. and at increasing SA by incubation of decreasing masses with a constant amount (7.5–8.5 MBq) of radioactivity (Table 2-1). A 3 h incubation period at 42 °C was employed to ensure high L.E., but shorter incubation periods may also be feasible (282). The maximum SA achievable with $^{111}$In based on a L.E. > 90% was 70.7 ± 3.2 MBq/μg and 65.3 ± 3.1 MBq/μg for panitumumab-MCP and panitumumab-DOTA, respectively. The maximum SA for labeling with $^{177}$Lu was 71.5 ± 9.2 MBq/μg and 46.2 ± 5.3 MBq/μg for panitumumab-MCP and panitumumab-DOTA, respectively. Because there were approximately two MCPs incorporating 26 DOTA or two DOTA directly conjugated to panitumumab, the maximum theoretical SA of panitumumab-MCP immunoconjugates labeled with $^{111}$In or $^{177}$Lu is 9.1-fold higher than that of panitumumab-DOTA, taking into account the higher MW of the polymer immunoconjugates. Thus, it may be possible to achieve even higher SA by labeling smaller masses of panitumumab-MCP (<0.1 μg) with $^{111}$In or $^{177}$Lu. The difference between the SA for labeling of the two immunoconjugates was revealed further when 1 μg was incubated with both $^{111}$In (7.8 MBq) and $^{177}$Lu (5.7 MBq). The SA of 1 μg of panitumumab-MCP labeled with 7.8 MBq of $^{111}$In and 5.7 MBq of $^{177}$Lu was 55.9 ± 4.8 MBq/μg and 20.5 ± 4.2 MBq/μg, respectively, while that of panitumumab-DOTA was 12.7-fold and 9.3-fold lower (4.4 ± 0.3 MBq/μg and 2.2 ± 0.3 MBq/μg, respectively). The 3.9-fold higher theoretical SA of carrier-free $^{111}$InCl$_3$ compared with $^{177}$LuCl$_3$ (1.6 × 104 MBq/μg vs 4.0 × 103 MBq/μg, respectively) and the higher amount of $^{111}$In than $^{177}$Lu used for dual-labeling (7.8 vs 5.7 MBq) contributed to the higher SA for $^{111}$In labeling.
Table 2-1. Labelling Efficiency (L.E.) and Specific Activity (SA) of Panitumumab-MCP and Panitumumab-DOTA with $^{111}$In or $^{177}$Lu$^a$.

<table>
<thead>
<tr>
<th>Immunoconjugates</th>
<th>Mass (µg)</th>
<th>$^{111}$In</th>
<th>177Lu</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L.E. (%)</td>
<td>SA (MBq/µg)</td>
</tr>
<tr>
<td>Panitumumab-MCP</td>
<td>100</td>
<td>99.5±0.2</td>
<td>0.1± 0.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>99±1</td>
<td>0.8± 0.0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>94±2</td>
<td>6.5± 0.4</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>95±2</td>
<td>70.7 ± 3.2</td>
</tr>
<tr>
<td>Panitumumab-DOTA</td>
<td>100</td>
<td>95±3</td>
<td>0.1±0.01</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>96±2</td>
<td>0.91±0.0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>92±3</td>
<td>6.6±0.6</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>92±2</td>
<td>65.3 ± 3.1</td>
</tr>
</tbody>
</table>

$^a$ Samples were labeled with 7.5–8.5 MBq of $^{111}$InCl$_3$ or $^{177}$LuCl$_3$ in 0.1 M HEPES buffer pH 5.0 for 3 h at 42 °C. Values reported are mean ± SEM (n = 6). L.E. was determined by ITLC-SG.
2.4.2 Hydrodynamic Radius

The hydrodynamic radius of panitumumab-MCP and panitumumab-DOTA were characterized by SEC-HPLC. A calibration curve was constructed by plotting the elution time of polyethylene glycols (PEG, 0.54–23.8 kDa, Polymer Laboratories) and protein standards (12.4–660 kDa, Sigma-Aldrich) against their corresponding hydrodynamic radius (Rh) (Table 2-2) and then plotting “retention time” vs “Rh”. The scaling relationship (Rh vs MW) of PEG (Figure 2-3) and protein standards were obtained from the literature (22-26). On the basis of this relationship, the resulting Rh vs retention time calibration curve was plotted (Figure 2-3). The unconjugated MCP and panitumumab-DOTA exhibited a peak Rh of 4.0 and 5.5 nm, respectively. Panitumumab-MCP showed a main peak corresponding to an Rh of 5.5 nm which was attributed to panitumumab and a shoulder peak corresponding to an Rh of 7.3 nm representing the MCP-conjugate. On the basis of the integration of the area under the main peak and shoulder peak, it was estimated that approximately 50% of panitumumab molecules were conjugated to MCP by assuming the refractive index increment (dn/dc) of both MCP and panitumumab are identical.
Figure 2-3. (A). The relationship of hydrodynamic radius (Rh) vs. molecular weight (MW) for PEG standards measured by intrinsic viscosity using a capillary viscometer (black squares) and by analytical ultracentrifugation (red diamonds), re-plotted from the literature. (B) Calibration curve of Rh vs. retention time for PEG standards by size exclusion chromatography (open squares: Rh obtained from the scaling relationship in Figure 2-3A and protein standards (blue triangles: Rh obtained from the literature S1-S5). The number shown near each data point is the MW (kDa) of the corresponding sample. (C) SEC profiles of panitumumab-DOTA, panitumumab-MCP and unconjugated MCP at 30 °C in water containing 0.2 M KNO₃, 25 mM NaH₂PO₄ (pH 8.5), and 200 ppm NaN₃. The corresponding Rh for the peak attributed to panitumumab-DTPA, MCP and the shoulder peak from panitumumab-MCP were obtained from panel (B).
Table 2-2. Hydrodynamic radii and retention times for PEG and protein standards by size exclusion chromatography

<table>
<thead>
<tr>
<th>MW (PEG) / kDa</th>
<th>$R_h$ (nm)$^a$</th>
<th>Retention time (min)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>5.1</td>
<td>13.84</td>
</tr>
<tr>
<td>22</td>
<td>5.0</td>
<td>13.79</td>
</tr>
<tr>
<td>10</td>
<td>3.2</td>
<td>14.44</td>
</tr>
<tr>
<td>8.0</td>
<td>2.8</td>
<td>14.59</td>
</tr>
<tr>
<td>5.7</td>
<td>2.3</td>
<td>14.83</td>
</tr>
<tr>
<td>3.8</td>
<td>1.8</td>
<td>15.51</td>
</tr>
<tr>
<td>2.0</td>
<td>1.3</td>
<td>16.32</td>
</tr>
<tr>
<td>1.4</td>
<td>1.0</td>
<td>16.65</td>
</tr>
<tr>
<td>0.95</td>
<td>0.8</td>
<td>17.18</td>
</tr>
<tr>
<td>0.54</td>
<td>0.6</td>
<td>17.94</td>
</tr>
</tbody>
</table>

Protein (MW / kDa)

<table>
<thead>
<tr>
<th>Protein (MW / kDa)</th>
<th>$R_h$ (nm)$^a$</th>
<th>Retention time (min)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroglobulin from bovine thyroid (660)</td>
<td>8.6</td>
<td>12.75</td>
</tr>
<tr>
<td>Apoferritin (440)</td>
<td>6.7</td>
<td>13.12</td>
</tr>
<tr>
<td>$\beta$-Amylase (200)</td>
<td>5.1</td>
<td>13.62</td>
</tr>
<tr>
<td>Alcohol Dehydrogenase (150)</td>
<td>4.5</td>
<td>13.81</td>
</tr>
<tr>
<td>Albumin, bovine serum (66)</td>
<td>3.7</td>
<td>14.06</td>
</tr>
<tr>
<td>Carbonic Anhydrase (29)</td>
<td>2.5</td>
<td>14.71</td>
</tr>
<tr>
<td>Cytochrome c (12.4)</td>
<td>1.9</td>
<td>15.49</td>
</tr>
</tbody>
</table>

$^a$: Hydrodynamic radii of PEG and protein standards were obtained from the literature (276-280). 
$^b$: Retention times were obtained by size-exclusion chromatography (SEC, Viscotek) on tandem ViscoGEL G4000PWXL and G2500PWXL columns maintained at 30 °C on a high performance liquid chromatography (HPLC) system equipped with a VE3210 UV/VIS detector and VE3580 refractive index detector. The eluent used was 0.2 M KNO₃, in 25 mM sodium phosphate buffer pH 8.5 containing 200 ppm NaN₃ and the flow rate was 1.0 mL/min delivered using a VE1122 Solvent Delivery System and a VE7510 GPC Degasser (Viscotek). D-glucose was used as an internal standard.
2.4.3 EGFR Immunoreactivity

Both panitumumab-MCP-\(^{177}\)Lu and panitumumab-DOTA-\(^{177}\)Lu exhibited saturable binding to EGFR-positive MDA-MB-468 human breast cancer cells and binding was significantly reduced in the presence of a 50-fold excess of unlabeled panitumumab (Figure 2-4). Since MDA-MB-468 cells express a high density of EGFR (1.3 \(\times\) 10\(^6\)) these cells were used to provide direct (saturation) binding curves that were more readily fitted to a 1-site binding model for comparison of the EGFR-binding properties of the RICs than PANC-1 PnCa cells which exhibit a 3.3-fold lower EGFR density. The dissociation constant (K\(_d\)) and maximum number of binding sites/cell (B\(_{max}\)) for binding of panitumumab-MCP-\(^{177}\)Lu to MDA-MB-468 cells were 2.2 \(\pm\) 0.6 nmol/L and 0.6 \(\pm\) 0.07 \(\times\) 10\(^6\) receptors/cell, respectively. The K\(_d\) and B\(_{max}\) for binding of panitumumab-DOTA-\(^{177}\)Lu to MDA-MB-468 cells were 1.0 \(\pm\) 0.4 nmol/L and 0.5 \(\pm\) 0.07 \(\times\) 10\(^6\) sites/cell. There were no significant differences between the K\(_d\) values (P = 0.06) for panitumumab-MCP-\(^{177}\)Lu and panitumumab-DOTA-\(^{177}\)Lu, indicating that both RICs exhibited high affinity binding to EGFR.
Figure 2-4. Binding of (A) panitumumab-DOTA-$^{177}$Lu or (B) panitumumab-MCP-$^{177}$Lu to EGFR positive MDA-468 human breast cancer cells in the absence (total binding; TB) or presence (nonspecific binding; NSB) of a 50-fold excess of unlabeled panitumumab. Specific binding (SB) was calculated by subtraction of NSB from TB. Values shown are the mean ± SEM (n = 3). Specifically bound cpm at saturation were converted to nmoles for calculation of the $B_{\text{max}}$. 
2.4.4 Radioimmunoconjugate Stability

Incubation of $^{177}$Lu- or $^{111}$In-labeled panitumumab-MCP or panitumumab-DOTA with a 500-fold excess of EDTA revealed no appreciable release of radioactivity from either of these RICs (Figure 2-5A), revealing high stability. SE-HPLC analysis of $^{177}$Lu- or $^{111}$In-labeled panitumumab-DOTA incubated in human plasma at 37 °C showed a single peak with retention time (tR) of 9.3 min up to 7 days, and the tR of this peak did not change over time (figure 2-5 B,C). SE-HPLC analysis of $^{111}$In- or $^{177}$Lu-labeled panitumumab-MCP was not feasible because the MCP interacts with the column matrix, resulting in poor elution characteristics. However, ultrafiltration of $^{177}$Lu- or $^{111}$In-labeled panitumumab-MCP (MW = 216 kDa) using a 100 kDa MWCO filter and measuring the percentage of radioactivity filtered vs retained showed no release of low MW radioactivity into the filtrate indicating stability of the RICs in human plasma under these same conditions.
Figure 2-5. (A) Radioimmunoconjugates (RICs) were challenged with a 500-fold excess of EDTA for 1 h at 25 °C and analyzed by ITLC-SG. No transchelation of radiometal from the radioimmunoconjugates to EDTA was observed as compared to the phosphate buffered saline (PBS) control samples. Values shown are mean ± SEM (n=3). SEM is not visible due to very small values. (B) SE-HPLC analysis of panitumumab-DOTA-\(^{111}\)In and (C) panitumumab-MCP-\(^{111}\)In in human plasma at 37°C on a BioSep-S-2000 column eluted with 0.1M NaH\(_2\)PO\(_4\) buffer (pH=7.0) at a flow rate of 0.8mL/min. There was no change in the chromatographic properties over an incubation time of 1 week (different colored lines).
The in vivo stability of panitumumab-MCP-\(^{111}\)In and panitumumab-DOTA-\(^{111}\)In was examined by measuring the percent of injected radioactivity retained in mice over time after i.v. injection. Both RICs were eliminated slowly from the body with 55.0 ± 0.9% and 59.7 ± 12.7% of the injected radioactivity eliminated over 72 h (P > 0.05). The only significant difference in the elimination of the RICs was at 4 h p.i. (P < 0.01; Figure 2-6A). Urinary and fecal excretion of radioactivity was also measured up to 72 h p.i. (Figure 2-6B). Most of the radioactivity eliminated from the body was excreted into the urine (34.1 ± 7.2% and 36.0 ± 1.6% for panitumumab-MCP-\(^{111}\)In and panitumumab-DOTA-\(^{111}\)In, respectively; P > 0.05), whereas fecal excretion was 4.9 ± 1.4% and 5.2 ± 2.0% for panitumumab-MCP-\(^{111}\)In and panitumumab-DOTA-\(^{111}\)In, respectively (P > 0.05).
Figure 2-6. (A) Percent of radioactivity retained in the whole body after i.v. (tail vein) injection of panitumumab-MCP-\(^{111}\)In or panitumumab-DOTA-\(^{111}\)In up to 72 h p.i. in NOD-scid mice engrafted s.c. with PANC-1 tumours. (B) Cumulative percent of radioactivity excreted in the urine or feces up to 72 h p.i. in NOD-scid mice with PANC-1 tumours injected i.v. with panitumumab-MCP-\(^{111}\)In or panitumumab-DOTA-\(^{111}\)In. * Statistically significant difference between groups (P < 0.01). Data shown are the mean ± SEM (n = 4).
2.4.5 Biodistribution and Imaging Studies

A biodistribution study comparing panitumumab-MCP-\(^{111}\)In and panitumumab-DOTA-\(^{111}\)In at 72 h p.i in NOD-scid mice bearing s.c. PANC-1 xenografts revealed an almost identical profile, except for the liver (Figure 2-7). The liver uptake of panitumumab-MCP-\(^{111}\)In was 2.9-fold significantly greater than that of panitumumab-DOTA-\(^{111}\)In (11.2 ± 1.0%ID/g vs 3.8 ± 1.3%ID/g; P = 0.001). There were no significant differences in tumour uptake between panitumumab-MCP-\(^{111}\)In and panitumumab-DOTA-\(^{111}\)In (6.9 ± 1.3%ID/g vs 6.6 ± 3.3%ID/g, respectively; P > 0.05). Preinjection of 100-fold excess (1 mg) of unlabeled panitumumab to block EGFR significantly decreased the tumour uptake of panitumumab-MCP-\(^{111}\)In and panitumumab-DOTA-\(^{111}\)In (0.02 ± 0.00%ID/g and 0.06 ± 0.02%ID/g, respectively). The tumour and normal tissue distribution of nonspecific IgG-MCP-\(^{111}\)In or IgG-DOTA-\(^{111}\)In were studied as additional controls to assess the specificity of tumour uptake of panitumumab RICs. The tumour uptake of IgG-DOTA-\(^{111}\)In (1.9 ± 0.3%ID/g) was 3.5-fold significantly lower than that of panitumumab-DOTA-\(^{111}\)In (P < 0.01). Unexpectedly, there was no significant difference between the tumour uptake of IgG-MCP-\(^{111}\)In (5.4 ± 0.3%ID/g) and panitumumab-MCP-\(^{111}\)In (6.9 ± 1.3%ID/g) (P > 0.05) in this study. There was also 2-fold significantly higher spleen uptake of IgG-DOTA-\(^{111}\)In (18.5 ± 1.7% ID/g) and IgG-MCP-\(^{111}\)In (20.6 ± 3.5% ID/g) compared with the corresponding panitumumab RICs (8.5 ± 3.5%ID/g vs 9.5 ± 0.6% ID/g). There was 3.1-fold significantly greater liver uptake of IgG-DOTA-\(^{111}\)In compared with panitumumab-DOTA-\(^{111}\)In (12.0 ± 1.7%ID/g vs 3.8 ± 1.3%ID/g, respectively; P < 0.001).
Figure 2-7. Tumour and normal tissue uptake [percent injected dose per gram (%ID/g)] with/without preinjection of an excess of unlabeled panitumumab in NOD-scid mice bearing s.c. PANC-1 xenografts at 72 h p.i. of (A) Panitumumab-DOTA-$^{111}$In or (B) Panitumumab-MCP-$^{111}$In. Also shown in each panel is the tumour and normal tissue uptake of nonspecific IgG-DOTA-$^{111}$In or IgG-MCP-$^{111}$In, respectively. * Statistically significant differences (P < 0.05). Data shown are mean ± SEM (n = 5).
The ability of the RICs to image s.c. PANC-1 tumours in NOD-scid mice was evaluated by performing SPECT/CT imaging studies (Figure 2-8). The images were in agreement with the results of biodistribution studies. SPECT/CT images showed tumour uptake as well as liver and spleen accumulation for both panitumumab-DOTA-\textsuperscript{111}In and panitumumab-MCP-\textsuperscript{111}In (Figure 2-8A,D). Tumours were not visualized with nonspecific IgG-DOTA-\textsuperscript{111}In or IgG-MCP-\textsuperscript{111}In (Figure 2-8C,F). There was decreased tumour uptake of the panitumumab RICs with preadministration of an 100-fold excess of unlabeled panitumumab to block EGFR compared with the images obtained without blocking (Figure 2-8B,E). Taken together, these images indicated EGFR-mediated tumour uptake of both panitumumab RICs.
Figure 2-8. Anterior (left) and lateral (right) whole-body SPECT/CT images of NOD-scid mice with s.c. PANC-1 human pancreatic xenografts (white circle) at 72 h p.i. of 37 MBq (10 μg) of panitumumab-DOTA-\(^{111}\)In or panitumumab-MCP-\(^{111}\)In without (A and D, respectively) or with (B and E, respectively) preinjection of an excess (1 mg) of unlabeled panitumumab 24 h prior to injection of the \(^{111}\)In-labeled RICs. SPECT/CT images of NOD-scid mice at 72 h p.i. of 25 MBq of nonspecific IgG-MCP-\(^{111}\)In or IgG-DOTA-\(^{111}\)In are shown in panels C and F, respectively. Also visualized on all image panels are the liver (long arrow) and spleen (short arrow). Images were adjusted to the same intensity. The units on the intensity bar are kBq.
2.5 Discussion

We describe for the first time in this study a novel MCP composed of a PGlu backbone that presents on average 13 pendant DOTA for complexing $^{111}$In or $^{177}$Lu and 10 PEG chains to minimize liver and spleen accumulation. The MCP was conjugated through a terminal HyNic functional group to panitumumab modified with S-4FB by reaction with lysine residues on the mAb. This bioconjugation strategy was first described by Schwartz et al. and patented in 2011.(17) The approach relies on formation of a stable bis-aryl hydrazone bond between the aromatic aldehyde on SF-4B and the terminal hydrazine on the polymer (Figure 2-1). Because formation of the bis-aryl hydrazone creates a chromophore that absorbs at 354 nm (Figure 2-2A), the bioconjugation reaction can be monitored spectrophotometrically in “real-time” and the reaction terminated when the desired number of polymers is conjugated to the mAb. In our case, a reaction time of 75 min resulted in conjugation of approximately two MCPs to panitumumab (Figure 2-2B). Polymer conjugation was confirmed by an upward shift in the band for panitumumab on SDS-PAGE (Figure 2-2C).

The ability of panitumumab-MCP to complex $^{111}$In or $^{177}$Lu to high SA was examined by determining the L.E. for decreasing masses (100 to 0.1 μg) incubated with a constant amount of radioactivity (7.5–8.5 MBq). Masses of panitumumab-MCP as small as 0.1 μg were labeled to >90% L.E. with $^{111}$In or $^{177}$Lu yielding a SA > 70 MBq/μg (Table 2-1). In contrast, while 0.1 μg of panitumumab-DOTA was labeled with $^{111}$In to a L.E. > 90%, the L.E. for $^{177}$Lu was 74%. A minimum of 1 μg of panitumumab-DOTA was needed to achieve a L.E. > 90% with $^{177}$Lu (Table 2-1). Thus, assuming a L.E. > 90%, the maximum SA practically achievable for panitumumab-DOTA labeled with $^{111}$In or $^{177}$Lu was 65.3 ± 3.1 MBq/μg and 46.2 ± 5.3 MBq/μg, respectively. Various factors affect the complexation of radiometals by metal chelators, including competing metal ions (283) and stable daughter products that result from decay of the parent radionuclide.(30) $^{111}$In is cyclotron-produced by the $^{111}$Cd(p,n)$^{111}$In reaction and decays by electron capture to stable $^{111}$Cd. $^{111}$In solutions contain Fe, Al, and Zn ions which along with the $^{111}$Cd decay product compete for binding to DOTA and decrease L.E. (283) $^{177}$Lu is reactor-produced by neutron irradiation of $^{176}$Lu and decays by β-particle emission to $^{177}$Hf. It has been reported that $^{177}$Hf does not interfere with $^{177}$Lu labeling of DOTA-conjugated peptides (284). However, $^{176}$Lu would be complexed by DOTA and the impact of this competition with $^{177}$Lu...
depends on the SA of $^{177}$Lu. In fact, it has been stated that there may be more than 3 times the number of atoms of $^{176}$Lu in $^{177}$Lu solutions (285). Our results showed that small masses of panitumumab-DOTA (0.1 μg) were more susceptible to competing metal interference in labeling with $^{177}$Lu than $^{111}$In (Table 2-1). Moreover, panitumumab-MCP, due to the 13 DOTA available for complexing radiometals is less effected by competing metal ions than panitumumab-DOTA which incorporated only two DOTA, resulting in higher SA. We have similarly found that conjugation of trastuzumab with an MCP incorporating 29 DTPA permitted labeling with $^{111}$In to a much higher SA than trastuzumab modified with only two DTPA per mAb (240).

An important consideration with MCP conjugation of mAbs is the effect of the polymer on receptor/antigen binding. The EGFR-binding properties of panitumumab-MCP-$^{177}$Lu and panitumumab-DOTA-$^{177}$Lu were compared using MDA-MB-468 human breast cancer cells that overexpress EGFR (1.3 × 10$^6$ receptors/cell). Both panitumumab-MCP-$^{177}$Lu and panitumumab-DOTA-$^{177}$Lu exhibited saturable binding to MDA-MB-468 cells that was displaced by an excess of unlabeled panitumumab, demonstrating specific EGFR-mediated binding (Figure 2-4). There were no significant differences in the EGFR binding affinity of both RICs ($K_d = 2.2 \pm 0.6$ nmol/L and $1.0 \pm 0.4$ nmol/L, respectively). These $K_d$ values were similar to those reported by our group for $^{123}$I-panitumumab ($K_d = 0.7 \pm 0.4$ nmol/L) (286). We did not measure the EGFR binding of $^{111}$In-labeled panitumumab-MCP or panitumumab-DOTA, but it is not expected that changing the radiometal complexed by DOTA would affect these properties.

$^{111}$In- or $^{177}$Lu-labeled panitumumab-MCP or panitumumab-DOTA were stable to loss of radiometal when challenged in vitro with a 500-fold excess of EDTA (Figure 2-5A) or when incubated in human plasma for up to 1 week (Figure 2-5BC). However, to investigate any potential instability that may occur in vivo due to proteolytic degradation of the PGLu backbone of the MCP or cleavage of the linkage between the MCP and panitumumab, the elimination of radioactivity from the body in mice and excretion into the urine and feces were compared up to 72 h p.i. of panitumumab-MCP-$^{111}$In or panitumumab-DOTA-$^{111}$In. There was relatively high whole body retention of radioactivity (55–60%) for both RICs (Figure 2-6A). Most radioactivity was eliminated in the urine with only a small amount in the feces, and there were no significant differences between panitumumab-MCP-$^{111}$In and panitumumab-DOTA-$^{111}$In (Figure 2-6B).
These results suggest that there was no instability of the RICs introduced by use of the MCP for labeling with $^{111}$In compared to DOTA.

The tumor and normal tissue localization of panitumumab-MCP-$^{111}$In and panitumumab-DOTA-$^{111}$In were compared at 72 h p.i. in NOD-scid mice with s.c. PANC-1 tumors (Figure 2-7). There were no significant differences in normal tissue localization between the two RICs, except for the liver which was 3-fold higher for panitumumab-MCP-$^{111}$In. We previously observed higher liver uptake of trastuzumab Fab conjugated to MCP with a PAm backbone that harbored residual negative charges after labeling with $^{111}$In (269). The MCP used in this study was designed with a PGlu backbone that exhibits lower liver uptake and the polymer was further modified with PEG chains to shield the residual negative charges on uncomplexed DOTA after labeling with $^{111}$In or $^{177}$Lu. It was expected that these design features would limit opsonization by plasma proteins that lead to liver and spleen uptake (256). However, further optimization of the MCP with additional PEG chains may be required to reduce the liver uptake of panitumumab-MCP-$^{111}$In (271). Liver uptake of the RICs is not due to EGFR binding since panitumumab does not bind to mouse EGFR. Liver uptake of panitumumab-DOTA-$^{111}$In in NOD-scid mice at 72 h p.i. was more than 4-fold lower than reported by Ogawa et al. for panitumumab-DTPA-$^{111}$In at 6 days p.i. in tumor-bearing nude mice (~40% ID/g) (287).

Importantly, the tumor uptake of panitumumab-MCP-$^{111}$In was not significantly different than panitumumab-DOTA-$^{111}$In (6.9 ± 1.3 vs 6.6 ± 3.3%ID/g, respectively; Figure 2-7; P > 0.05). Furthermore, tumor uptake was EGFR-mediated because preadministration of a 100-fold excess of unlabeled panitumumab 24 h prior to injection of the RICs greatly reduced the tumor uptake of panitumumab-MCP-$^{111}$In and panitumumab-DOTA-$^{111}$In to 0.02 ± 0.00%ID/g and 0.06 ± 0.02%ID/g, respectively. The relatively low tumor uptake (<10% ID/g) of the RICs may be due to a moderate EGFR expression level on PANC-1 cells ($4 \times 10^5$ EGFR/cell), despite overexpression compared with normal pancreas cells. In a previous study, we found that the tumor uptake of $^{111}$In-labeled anti-EGFR mAb528 at 72 h p.i. in mice with MCF-7 human breast cancer xenografts ($1.5 \times 10^4$ EGFR/cell) was 8.4 ± 1.6% ID/g at 72 h p.i., while uptake in MDA-MB-468 tumors ($1.3 \times 10^6$ EGFR/cell) was 15.3 ± 2.5% ID/g (288). Other factors such as tumor vascularity may affect the uptake of the RICs in PANC-1 tumors since one study
reported a discordance between EGFR expression level in tumour xenografts in mice and the uptake of $^{89}$Zr-labeled anti-EGFR, cetuximab (289).

Interestingly, there was a major decrease in radioactivity in the blood and most normal tissues for these RICs associated with preinjection of an excess of panitumumab. The reasons are not known. However, Michel et al. reported that scid mice have very low levels of circulating IgG2a which causes rapid blood clearance of injected IgG2a due to binding to neonatal Fc receptors (FcRn) particularly in the spleen (290). Lower uptake of injected IgG2a compared with IgG1 in scid mice was found for liver, kidneys, lungs and muscle. Since panitumumab is a human IgG2a, it may similarly exhibit a rapid elimination from the blood and normal tissues in NOD-scid mice, which have scid background. FcRn rescues immunoglobulins from degradation in lysosomes, and Bleeker et al. showed that administration of an excess of IgG in C57BL/6 mice promoted elimination of an IgG1 mAb from the blood by more than 40% at 72 h p.i. (291). This may explain the greater elimination of radioactivity from the blood and normal tissues in NOD-scid mice preadministered an excess of panitumumab (1 mg) compared with mice receiving only small amounts (10 μg) of the RICs alone. Swiercz et al. reported that FcRn were present only at very low or undetectable levels on tumour cell lines (although PANC-1 was not examined) (292). Thus, we interpret the decreased uptake of radioactivity in PANC-1 tumours for the RICs with preadministration of an excess of panitumumab as related to blocking EGFR and not FcRn on these tumours.

To further examine the EGFR-mediated tumour uptake of panitumumab RICs, we compared their biodistribution to nonspecific IgG modified with MCP or DOTA and labeled with $^{111}$In (Figure 2-7). The tumour uptake of IgG-DOTA-$^{111}$In (1.9 ± 0.3%ID/g) was 3.5-fold significantly lower than panitumumab-DOTA-$^{111}$In as expected. Unexpectedly, there was no significant difference between the tumour uptake of IgG-MCP-$^{111}$In (5.4 ± 0.3% ID/g) and panitumumab-MCP-$^{111}$In (6.9 ± 1.3%ID/g; P > 0.05). This may be due to a larger hydrodynamic radius for IgG-MCP-$^{111}$In compared with IgG-DOTA-$^{111}$In, analogous to that found for panitumumab-MCP vs DOTA-panitumumab (7.1 nm vs 5.1 nm; respectively; Figure 2-3). This increase in molecular size may encourage nonspecific uptake of IgG-MCP-$^{111}$In into tumours via the enhanced permeability and retention (EPR) effect (293). We previously found that uptake of $^{111}$In-labeled nonspecific IgG into human breast cancer xenografts via the EPR effect in some cases was
sufficient for tumour imaging, but the effect varied dependent on the tumour xenograft model (294). Nonetheless, EGFR-binding likely contributes to the tumour uptake of panitumumab-MCP-\(^{111}\)In because saturation receptor-binding assays (Figure 2-4) revealed no significant differences in EGFR-binding affinity between panitumumab-MCP-\(^{177}\)Lu and panitumumab-DOTA-\(^{177}\)Lu. We did not study the tumour and normal tissue uptake of panitumumab-MCP-\(^{177}\)Lu or panitumumab-DOTA-\(^{177}\)Lu, but \(^{111}\)In- and \(^{177}\)Lu-labeled mAbs exhibit similar biodistribution properties (295, 296). Finally, microSPECT/CT at 72 h p.i. of panitumumab-MCP-\(^{111}\)In and panitumumab-DOTA-\(^{111}\)In visualized s.c. PANC-1 human PnCa xenografts in NOD-scid mice (Figure 2-8). Tumours were not visualized in mice preadministered an excess of unlabeled panitumumab to block EGFR, or in mice injected with nonspecific IgG RICs. The higher liver uptake of the polymer RICs was also apparent on the images.

2.6 Conclusions

Panitumumab was modified with a novel MCP that presents multiple DOTA chelators enabling stable and efficient complexation of \(^{111}\)In or \(^{177}\)Lu. The EGFR binding affinity of panitumumab was preserved following MCP conjugation. Panitumumab-MCP-\(^{111}\)In exhibited EGFR-mediated tumour uptake in mice with s.c. PANC-1 human PnCa xenografts that was not significantly different than panitumumab-DOTA-\(^{111}\)In. Normal tissue distribution was similar except for the liver which was higher for the polymer radioimmunoconjugates. The ability to dual-label panitumumab-MCP with \(^{111}\)In and \(^{177}\)Lu allows SPECT imaging as well as RIT exploiting the Auger electrons emissions of \(^{111}\)In and β-particles emitted by \(^{177}\)Lu, thus enabling a theranostic approach.
Chapter 3: Radioimmunotherapy of PANC-1 Human Pancreatic Cancer Xenografts in NRG Mice with Panitumumab Modified with Metal-Chelating Polymers (MCP) Complexed to $^{177}$Lu
This chapter represents a reprint of Radioimmunotherapy of PANC-1 Human Pancreatic Cancer Xenografts in NRG Mice with Panitumumab Modified with Metal-Chelating Polymers (MCP) Complexed to $^{177}$Lu. All experiments were designed and carried out by Sadaf Aghevlian. Dosimetry was performed with assistance from Dr. Zhongli Cai. The synthesis and characterization of metal chelating polymer were performed by Dr. Yijie Lu at the chemistry department, at University of Toronto.
3. Abstract

Our aim was to evaluate the effectiveness and normal tissue toxicity of radioimmunotherapy (RIT) of s.c. PANC-1 human pancreatic cancer (PnCa) xenografts in NRG mice using anti-EGFR panitumumab linked to metal-chelating polymers (MCP) that present 13 DOTA chelators to complex the β-emitter, $^{177}$Lu. The clonogenic survival (CS) of PANC-1 cells treated in vitro with panitumumab-MCP-$^{177}$Lu (0.3-1.2 MBq) and DNA double-strand breaks (DSBs) in the nucleus of these cells were measured by confocal immunofluorescence microscopy for γ-H2AX. Subcellular distribution of radioactivity for panitumumab-MCP-$^{177}$Lu was measured and absorbed doses to the cell nucleus calculated. Normal tissue toxicity was assessed in non-tumour bearing NRG mice by monitoring body weight, complete blood cell counts (CBC) and serum alanine aminotransferase (ALT) and creatinine (Cr) after i.v. injection of 6 MBq (10 μg) of panitumumab-MCP-$^{177}$Lu. RIT was performed in NRG mice with s.c. PANC-1 tumours injected i.v. with 6 MBq (10 μg) of panitumumab-MCP-$^{177}$Lu. Control mice received non-specific human IgG-MCP-$^{177}$Lu (6 MBq; 10 μg), unlabeled panitumumab (10 μg) or normal saline. The tumour growth index (TGI) was compared. Tumour and normal organ doses were estimated based on biodistribution studies. Panitumumab-MCP-$^{177}$Lu reduced the CS of PANC-1 cells in vitro by 7.7-fold at the highest amount tested (1.2 MBq). Unlabeled panitumumab had no effect on the CS of PANC-1 cells. γ-H2AX foci were increased by 3.8-fold by panitumumab-MCP-$^{177}$Lu. Panitumumab-MCP-$^{177}$Lu deposited 3.84 Gy in the nucleus of PANC-1 cells. Administration of panitumumab-MCP-$^{177}$Lu (6 MBq; 10 μg) to NRG mice caused no change in body weight, CBC or ALT and only a slight increase in Cr compared to NRG mice treated with normal saline. Panitumumab-MCP-$^{177}$Lu strongly inhibited tumour growth in NRG mice (TGI = 2.3 ± 0.2) compared to normal saline treated mice (TGI = 5.8 ± 0.5; P<0.01). Unlabeled panitumumab had no effect on tumour growth (TGI = 6.0 ± 1.6; P>0.05). The absorbed dose of PANC-1 tumours was 12.3 Gy. The highest normal organ doses were absorbed by the pancreas, liver, spleen, and kidneys. We conclude that EGFR-targeted RIT with panitumumab-MCP-$^{177}$Lu was able to overcome resistance to panitumumab in KRAS mutant PANC-1 tumours in NRG mice and may be a promising approach to treatment of PnCa in humans.
Graphical abstract
3.1 Introduction

Pancreatic cancer (PnCa) is a rapidly fatal malignancy due to its often advanced stage at diagnosis combined with the limited treatment options (297). Surgery remains the only hope for long-term survival, but only 10-15% of patients are candidates for surgical resection due to local invasion or distant metastasis (298). Even in patients who undergo surgical resection with curative intent followed by chemotherapy, the median survival is 17-23 months. For patients who are not candidates for surgery due to locally advanced PnCa, the median survival is 8-14 months and is 4-6 months for patients with metastatic PnCa (299). New therapeutic strategies are needed for patients with PnCa. Stereotactic body radiation therapy (SBRT) has proven effective for controlling local progression of PnCa providing freedom from local progression (FFLP) for as long as a year in 80% of patients with locally advanced PnCa, but patients ultimately die of metastatic disease (300). Radioimmunotherapy (RIT) which offers an opportunity to selectively treat disseminated PnCa at any location in the body with radiation, may improve patient outcome in patients with metastatic disease. RIT with the humanized monoclonal antibody (mAb), clivatuzumab (Immunomedics, Morris Plains, NJ) targeting the cell surface mucin MUC1 (301) labeled with the β-emitter, $^{90}$Y [$E_{\beta_{\text{max}}}=2.2$ MeV (100%); $t_{1/2}=2.7$ d] showed promising results (partial remission or stable disease) in a Phase I/II trial combined with gemcitabine for treatment of metastatic PnCa (302). Improved survival was found in patients with metastatic PnCa in a subsequent Phase 1 trial that combined $^{90}$Y-clivatuzumab with gemcitabine chemotherapy compared to RIT alone (7.8 vs. 3.4 months) (303). However, interim analysis of a Phase 3 trial (PANCRIT-1) that randomized patients to RIT + gemcitabine and best supportive care (BSC) or gemcitabine and BSC alone did not demonstrate improved survival in the RIT arm (https://clinicaltrials.gov/ct2/show/NCT01956812) and the trial was discontinued. However, patients in the PANCRIT-1 trial had metastatic PnCa that had progressed after at least two prior chemotherapy regimens. Thus, there remains the potential that earlier treatment of PnCa in patients with lower tumour burden may improve outcome. In addition, there is an emerging interest in the use of RIT to eradicate small tumours and minimal residual disease to prevent disease progression after other forms of cancer therapy (304). The shorter range (2 mm) β-particles emitted by $^{177}$Lu [$t_{1/2}=6.7$ d; $E_{\beta_{\text{max}}}=0.5$ MeV (78.6%); $E_{\beta_{\text{max}}}=0.38$ MeV (9.1%); $E_{\beta_{\text{max}}}=0.18$ MeV (78.6%)] may be more appropriate for RIT of small tumours, while the higher
energy \([\text{E} \beta_{\text{max}} = 2.2 \text{ MeV (100\%)}]\) and longer range (12 mm) β-particles emitted by \(^{90}\text{Y}\) are more useful for treatment of larger tumours. Modeling has revealed that the optimal tumour diameter for treatment with \(^{177}\text{Lu}\) is ~2 mm, while for \(^{90}\text{Y}\), the ideal tumour diameter for RIT is 34 mm (305). Indeed, de Jong et al. reported that co-treatment with a mixture of \(^{177}\text{Lu}-\) and \(^{90}\text{Y}\)-labeled DOTATATE radiopeptides was more effective than either radiopeptide alone in prolonging the survival of rats with both large and small somatostatin receptor-positive CA20948 s.c. pancreatic neuroendocrine tumours (306).

Panitumumab (Vectibix; Amgen, Thousand Oaks, CA) is a human IgG\(_2\) mAb that binds the epidermal growth factor receptor (EGFR) (307). EGFR are overexpressed on PnCa (>90% of cases) (308). Naked anti-EGFR mAbs have not proven effective for treatment of PnCa (309, 310) since there is often downstream KRAS mutation (311), but this is not limiting for RIT, since the mAbs are used only to target radioactivity to tumours. We recently reported novel radioimmunoconjugates (RICs) for RIT of PnCa consisting of panitumumab linked to metal-chelating polymers (MCP) that complex \(^{177}\text{Lu}\) or \(^{111}\text{In}\) (312). The MCP are composed of a polyglutamide backbone with 10 pendant polyethylene glycol (PEG) chains with the aim to inhibit liver and spleen uptake of the RICs, and 13 DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) chelators that strongly complex \(^{177}\text{Lu}\) or \(^{111}\text{In}\) (312). These polymer immunoconjugates have theranostic capability since they may be labeled simultaneously to high specific activity (SA) with \(^{177}\text{Lu}\) and \(^{111}\text{In}\). The β-particle emissions of \(^{177}\text{Lu}\) and Auger electrons emitted by \(^{111}\text{In}\) enable RIT, while the γ-emissions of \(^{111}\text{In}\) \([E_{\gamma} = 171 \text{ keV (90\%)} \text{ and } E_{\gamma} = 245 \text{ keV (94\%)}]\) allow single photon emission computed tomography (SPECT).

In the current study, we examine the cytotoxicity of panitumumab-MCP-\(^{177}\text{Lu}\) in vitro on EGFR-positive PANC-1 human PnCa cells and its ability to inflict lethal DNA double-strand breaks (DSBs) in the nucleus of these cells and estimate the absorbed doses in the cell nucleus. We further report for the first time the effectiveness and normal tissue toxicity of RIT of PANC-1 tumours in NOD-Rag1\(^{-/-}\)-IL2RgammaC-null (NRG) mice with panitumumab-MCP-\(^{177}\text{Lu}\), and estimate the absorbed doses in tumours and normal organs. We hypothesized that RIT with panitumumab-MCP-\(^{177}\text{Lu}\) would be effective for treatment of PANC-1 tumours at administered amounts that are non-toxic to normal tissues.
3.2 Materials and Methods

3.2.1 Cell culture and tumour xenografts

PANC-1 human pancreatic adenocarcinoma cells (4.0 × 10^5 EGFR/ cell) (313) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). PANC-1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (Gibco Life Technologies; Burlington, ON, Canada) supplemented with 1% penicillin and streptomycin and 10% fetal bovine serum (Gibco-Invitrogen, Thermo Fisher Scientific, Waltham, MA). Tumour xenografts (8-10 mm diameter) were established in female NOD-Rag1−/−IL2RgammaC-null (NRG) mice (University Health Network, Toronto, ON) by subcutaneous (s.c.) inoculation of 4 × 10^6 PANC-1 cells in serum free DMEM (100 μL) into the left flank.

3.2.2 Radioimmunoconjugates

The synthesis of hydrazino nicotinamide (HyNic) end-capped HyNic-MCP presenting 13 DOTA chelators and 10 PEG chains to minimize liver and spleen uptake was previously reported (271). Panitumumab-MCP immunoconjugates were constructed by cross-linking panitumumab IgG to two HyNic-MCPs through an N-succinimidyl-4-formylbenzamide (S-4FB) moiety introduced into panitumumab and stored in 0.1 M HEPES buffer, pH 5.5. Immunoconjugates (5-70 μg; 4-12 μL) were radiolabeled by incubation with ^{177}LuCl_3 (0.75-49 MBq; 37 MBq/μL; PerkinElmer, Waltham, MA) at 42 °C for 2 h (271). The final radiochemical purity of the RICs was >95% determined by instant thin-layer silica gel chromatography (ITLC-SG; Pall) developed in 0.1 M sodium citrate, pH 5.5. We previously reported that the K_D for binding of panitumumab-MCP complexed to ^{111}In to EGFR-positive MDA-MB-468 human breast cancer cells was 2.2 ± 0.6 nmol/L (312). Non-specific human IgG (hIgG; Sigma-Aldrich, St. Louis, MO, Product No. 14506) was similarly modified with MCP and labeled with ^{177}Lu. The RICs were shown to be stable to loss of radiometal by challenge with an excess of ethylenediaminetetraacetic acid (EDTA) and in plasma at 37 °C (312).

3.2.3 In vitro cytotoxicity studies

The clonogenic survival (CS) of PANC-1 cells treated in vitro with panitumumab-MCP-^{177}Lu was studied. Briefly, 2 × 10^5 cells were treated with 2.5 nmol/L (50-fold molar excess) of
panitumumab-MCP labeled with 0.3, 0.6 or 1.2 MBq of \(^{177}\text{Lu} \) [SA = 46.7, 93.5, 187.0 MBq/nmole] in 500 μL of growth medium in 24 well plates for 16 h. Controls consisted of cells exposed to unlabeled panitumumab-MCP or to growth medium alone. Approximately 700 cells were then seeded into 6 well plates and cultured for 12 d. Surviving colonies were stained with methylene blue and colonies with >50 cells were counted. The plating efficiency (PE) was determined by dividing the number of colonies formed by the number of cells seeded. The CS was calculated by dividing the PE for treated cells by that for untreated cells (314).

Induction of DNA double-strand breaks (DSBs) in PANC-1 cells caused by panitumumab-MCP-\(^{177}\text{Lu} \) was assessed by immunofluorescence confocal microscopy for phosphorylated histone-2A (γ-H2AX) which accumulates at sites of DSBs in the cell nucleus (315). Briefly, \(2 \times 10^5\) PANC-1 cells were seeded onto glass coverslips (Ted Pella, Redding, CA) in 24-well plates and cultured overnight at 37 °C. The cells were then treated with panitumumab-MCP-\(^{177}\text{Lu} \) as described earlier. Controls consisted of cells exposed to unlabeled panitumumab-MCP or to growth medium alone. Immunostaining for γ-H2AX and analysis of confocal microscopy images for the density of γ-H2AX foci per nucleus area followed previously reported procedures (316). Normalization of γ-H2AX foci per nucleus area rather than the number of foci in the nucleus minimizes differences in cell nucleus size between cells.

3.2.4 Subcellular distribution and microdosimetry

The subcellular distribution of panitumumab-MCP-\(^{177}\text{Lu} \) in PANC-1 cells was measured to estimate the absorbed doses in the cell nucleus (microdosimetry). Briefly, \(2 \times 10^5\) cells were cultured overnight in wells in a 6-well plate. A 50-fold molar excess of panitumumab-MCP-\(^{177}\text{Lu} \) (1.2 MBq; 2.5 nmol/L) were added to the wells and the plates were incubated for 1, 4, 8, or 24 h at 37 °C in the absence or presence of excess of unlabeled panitumumab to block EGFR to confirm the EGFR specificity of binding of panitumumab-MCP-\(^{177}\text{Lu} \) to PANC-1 cells. Cell fractionation studies were also performed for cells incubated with non-specific hIgG-MCP-\(^{177}\text{Lu} \) (1.2 MBq; 2.5 nmol/L). The medium was removed and the cells which were exposed to panitumumab-MCP-\(^{177}\text{Lu} \) were fractionated to determine the dose to the cell nucleus from the cell surface, cytoplasm and nucleus source compartments. The cells were first rinsed with phosphate-buffered saline (PBS), pH 7.5, then the proportion of internalized cell-bound radioactivity was determined by displacing the cell-surface \(^{177}\text{Lu} \) with 1 mL of 200 mM sodium
acetate/500 mM NaCl, pH 2.5 at room temperature (RT). This procedure was repeated twice and the cell membrane fractions were combined and measured in a γ-counter. The cell membrane was then lysed twice in 500 μL of Nuclei EZ Lysis buffer (Sigma-Aldrich) on ice for 60 min. A cell scraper was used to gently detach cells from each well and the cells were then transferred to 1.5 mL Eppendorf tubes. The tubes were centrifuged at 3,000 × g for 5 min to separate nuclear and cytoplasmic radioactivity (supernatant). The radioactivity in the nucleus, membrane and cytoplasm fractions was measured in a γ-counter and the percentage of radioactivity in each fraction (relative to the total cell-bound \(^{177}\)Lu) was calculated. This cell fractionation procedure has been previously validated by our group to yield pure cytoplasmic and nuclear fractions (317).

The absorbed dose in the nucleus (D) of a PANC-1 cell after exposure to 1.2 MBq of panitumumab-MCP-\(^{177}\)Lu (2.5 nmol/L) was estimated based on the cumulative amount of radioactivity (\(\bar{A}\)s) on the cell surface, in the cytoplasm and in the cell nucleus as well as non cell-bound radioactivity in the surrounding culture medium. \(\bar{A}\)s was calculated from the area under the curve from 0 to 24 h (AUC\(_{0-24h}\); Bq × sec) using Prism Ver. 4.0 software (GraphPad). The dose to the nucleus was estimated as \[D = \bar{A}\text{s} \times S\], and the S-values (Gy/Bq × sec) were calculated using MCNP Monte Carlo code Ver. 5 (Los Alamos National Laboratory, Los Alamos, NM). A mean cell radius of 17.1 μm and nucleus radius of 7.0 μm were used. These were measured by fluorescence microscopy of PANC-1 cells and Image J software (National Institutes of Health, Bethesda, MD) after staining of the nucleus with 4',6-diamidino-2-phenylindole (DAPI) (221). The absorbed doses were also calculated for PANC-1 cells exposed to panitumumab-MCP-\(^{177}\)Lu co-incubated with a 50-fold molar excess of unlabeled panitumumab to block EGFR, or non-specific hIgG-MCP-\(^{177}\)Lu (1.2 MBq; 2.5 nmol/L).

3.2.5 Evaluation of normal tissue toxicity

To identify a non-toxic dose for subsequent RIT studies, groups of 5 healthy, non-tumour bearing female NRG mice were injected i.v. (tail vein) with 6.0 MBq (10 μg) of panitumumab-MCP-\(^{177}\)Lu or non-specific hIgG-MCP-\(^{177}\)Lu in 100 μL of normal saline. These doses were selected based on a previous study that reported that 8.0 MBq of \(^{177}\)Lu-labeled RICs were administered safely without major toxicity to NRG mice (no loss in body weight) (318). NRG mice were selected for these studies and for RIT studies, since unlike NOD/SCID mice, NRG mice do not harbor a germ line defect in DNA repair (319) which renders NOD/SCID mice...
unusually sensitive to radiation. Control mice received injections of normal saline or panitumumab. Body weight was monitored every 2-4 d for 14 d. The mice were then sacrificed and samples of blood were collected into EDTA-coated microtubes and centrifuged to separate serum for biochemistry [serum alanine aminotransferase (ALT) and creatinine (Cr)] analyses. ALT and Cr were measured using Infinity Creatinine or ALT clinical chemistry kits (Fisher Diagnostics, Middletown, VA). Complete blood cell (CBC) counts, hematocrit (HCT) and hemoglobin (Hb) were measured on a HemaVet 950FS instrument (Drew Scientific, Miami Lakes, FL). The 14 d period for assessing normal tissue toxicity was selected based on the requirements of Health Canada for acute toxicity testing of new drugs (320).

3.2.6 Radioimmunotherapy studies

The tumour-growth inhibitory effects of panitumumab-MCP-\textsuperscript{177}Lu were studied in groups of NRG mice (n=7) with s.c. PANC-1 xenografts. The number of mice in each group (7) was sufficient to detect a minimum difference of 0.8 in the mean tumour growth index (TGI) with a standard deviation of 0.5, an $\alpha$-value = 0.05 and power, $\beta$-value = 0.80 (https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html). Mice received a single i.v. injection (tail vein) of 6.0 MBq (10 μg) of panitumumab-MCP-\textsuperscript{177}Lu in 100 μL of normal saline. Control groups of NRG mice were injected with unlabeled panitumumab (10 μg) or normal saline. Tumour length (mm) and width (mm) were measured using calipers every 2-3 days until the humane endpoint of the animal care protocol was reached (tumour diameter $>$15 mm). The tumour volume ($V$; mm$^3$) was calculated as $V = $length $\times$ width$^2 \times 0.5$ (321). The TGI was calculated by dividing the tumour volume at each time point by the tumour volume at the initiation of treatment. Similarly, the body weight index (BWI) was estimated by dividing the body weight at each time point by the pre-treatment body weight.

3.2.7 Tumour and normal organ dosimetry

The absorbed doses in the tumour and normal organs in NRG mice with s.c. PANC-1 xenografts after i.v. injection of 6.0 MBq (10 μg) of panitumumab-MCP-\textsuperscript{177}Lu were estimated. Groups of 5 mice were injected with RICs, then at selected time points ranging from 24 to 168 h p.i., the mice were sacrificed by cervical dislocation under anesthesia, and the tumour and samples of normal tissues including blood were collected, weighed, and counted in a γ-counter. Tumour and normal
organ uptake were expressed as MBq/g, then converted to MBq/organ by multiplying by previously determined standard weights for mouse organs (322). The cumulative radioactivity in the tumour and normal source organs from 0-168 h ($\bar{A}_{0-168h}$) was estimated using Prism Ver. 4.0 software (GraphPad, San Diego, CA) from the AUC$_{0-168h}$ (MBq × h) determined in biodistribution studies. The cumulative radioactivity from 168 h to infinity ($\bar{A}_{168h-\infty}$) was calculated by dividing the radioactivity at the final measured time point by the elimination rate constant for $^{177}$Lu (0.004345 h$^{-1}$), thus assuming that further elimination occurs only by radioactive decay without additional biological elimination. We have previously applied this assumption to estimate the absorbed doses for other radiolabeled mAbs (322). The $\bar{A}_{0-\infty}$ values of each source organ (MBq × h) were converted to Bq x sec by multiplying by $(3.6 \times 10^3 \text{ secs/h})(10^6 \text{ Bq/MBq})$. Then, these $\bar{A}_{0-\infty}$ values were multiplied by the respective S-value (Gy/Bq × sec) of the source to target organs for $^{177}$Lu using published mouse S-values (323), then summing up all the absorbed doses to the target organ from all source organs. The dose delivered to the tumour was estimated based on S-values of the sphere model in OLINDA/EXM, using the average measured tumour volume at the time of treatment (324).

3.3 Statistical analysis

Results were expressed as mean ± SEM. Statistical significance was tested using an unpaired two-tailed Student’s t-test (P<0.05) or in multiple comparisons by ANOVA (F-test; P<0.05) using Prism Ver. 4.0 software (GraphPad).

3.4 Results

3.4.1 In vitro cytotoxicity studies

There was no effect of unlabeled panitumumab-MCP on the CS of PANC-1 cells (Figure 3-1a). CS was decreased with exposure to increasing activity of panitumumab-MCP-$^{177}$Lu. At the highest radioactivity of panitumumab-MCP-$^{177}$Lu studied (1.2 MBq), the CS of PANC-1 cells was decreased by 7.4-fold compared to untreated cells. Exposure of PANC-1 cells to 0.6 MBq of panitumumab-MCP-$^{177}$Lu reduced the CS by 9.0-fold, but this was not significantly different than cells treated with 1.2 MBq. The CS of cells exposed to 0.3 MBq of panitumumab-MCP-$^{177}$Lu was reduced by 1.9-fold, and this was significantly lower than cells exposed to 0.6 or 0.9
MBq. There were no significant differences in the density of γ-H2AX foci in the nucleus of PANC-1 cells exposed to unlabeled panitumumab-MCP compared to untreated cells (Figure 3-1b). Similarly, no differences in γ-H2AX foci were found in cells exposed to 0.3 MBq of panitumumab-MCP-177Lu compared to cells exposed to these control treatments. However, PANC-1 cells exposed to 0.6 MBq or 1.2 MBq of panitumumab-MCP-177Lu exhibited a significantly greater density of γ-H2AX foci. At the highest amount tested (1.2 MBq), γ-H2AX foci were increased by 3.8-fold by panitumumab-MCP-177Lu. Confocal immunofluorescence microscopy for γ-H2AX (Figure 3-1c) showed more γ-H2AX foci in the nucleus of cells treated with panitumumab-MCP-177Lu than for cells exposed to unlabeled immunoconjugates or untreated cells.
Figure 3-1. (A) Clonogenic survival of PANC-1 human pancreatic cancer cells exposed to increasing radioactivity of panitumumab-MCP-\(^{177}\)Lu (0.3-1.2 MBq, 2.5 nmol/L) compared to untreated cells (0.0 MBq). (B) Density of \(\gamma\)-H2AX foci in the nucleus of PANC-1 cells representing sites of DNA double-strand breaks (DSBs) following exposure to increasing amounts of panitumumab-MCP-\(^{177}\)Lu or to unlabeled radioimmunoconjugates (RICs) or in untreated cells. (C) Immunofluorescence confocal microscopy images of PANC-1 cells exposed to these treatments. Bright foci in the nucleus of the cells (counterstained blue with DAPI) are \(\gamma\)-H2AX foci, which are more prevalent in cells treated with panitumumab-MCP-\(^{177}\)Lu compared to untreated cells or cells exposed to unlabeled RICs.
3.4.2 Subcellular localization and microdosimetry

There was a time-dependent increase in the percentage of radioactivity bound to PANC-1 cells after incubation with panitumumab-MCP-\(^{177}\)Lu for 1, 4, 8 or 24 h at 37 °C (Figure 3-2a). The percentage of cell-bound radioactivity for non-specific hIgG-MCP-\(^{177}\)Lu or panitumumab-MCP-\(^{177}\)Lu in the presence of excess of unlabeled panitumumab at 24 h was significantly lower than for panitumumab-MCP-\(^{177}\)Lu (1.21 ± 0.18% or 0.35 ± 0.01% vs. 4.16 ± 0.17%; P<0.001 respectively) indicating the EGFR-specificity of binding of panitumumab-MCP-\(^{177}\)Lu RICs. The percentage of the total radioactivity bound to the cells that was localized on the cell membrane, in the cytoplasm or in the nucleus was measured by subcellular fractionation of cells treated with panitumumab-MCP-\(^{177}\)Lu (Figure 3-2b). Cell-membrane radioactivity decreased over 24 h, representing internalization of the RICs, while cytoplasmic and nuclear radioactivity increased.
Figure 3-2. (A) Percentage of radioactivity bound to PANC-1 cells at selected times after incubation with panitumumab-MCP-\(^{177}\)Lu in the absence or presence of excess of unlabeled panitumumab or with non-specific hIgG-MCP-\(^{177}\)Lu. (B) Percentage of total cell-bound radioactivity which was localized on the cell membrane, in the cytoplasm or in the nucleus of PANC-1 cells at selected times after incubation with panitumumab-MCP-\(^{177}\)Lu.
The cumulative radioactivity in the cell surface, cytoplasm and nucleus source compartments ($A_s$) as well as non-cell bound radioactivity in the culture medium was used to estimate the absorbed dose in the nucleus of a PANC-1 cell exposed to the maximum radioactivity (1.2 MBq) of panitumumab-MCP-\textsuperscript{177}Lu (Table 3-1). A total of 3.87 Gy of radiation was deposited in the nucleus of PANC-1 cells when exposed to panitumumab-MCP-\textsuperscript{177}Lu (1.2 MBq). The absorbed dose was deposited mainly by \textsuperscript{177}Lu present on the cell surface, followed by cytoplasmic \textsuperscript{177}Lu, then \textsuperscript{177}Lu in the nucleus. An additional 25% of the total absorbed dose in the nucleus of PANC-1 cells was due to non-cell bound \textsuperscript{177}Lu in the culture medium through a cross-fire effect, during the 24 h incubation period prior to recovering and seeding the cells for clonogenic assay. The absorbed dose deposited in the nucleus of PANC-1 cells exposed to panitumumab-MCP-\textsuperscript{177}Lu was 2.7-fold higher than for panitumumab-MCP-\textsuperscript{177}Lu co-incubated with a 50-fold molar excess of unlabeled panitumumab to block EGFR (1.46 Gy), or non-specific hIgG-MCP-\textsuperscript{177}Lu (2.06 Gy) (Table 3-1). A higher proportion of the dose deposited by these control treatments was due to radioactivity present in the growth medium during the 16 h incubation period than for panitumumab-MCP-\textsuperscript{177}Lu.
Table 3-1. Absorbed doses in the nucleus of PANC-1 cells in vitro by panitumumab-MCP-\textsuperscript{177}Lu \textsuperscript{a}

<table>
<thead>
<tr>
<th>Source compartment</th>
<th>(\bar{\text{A}}_s) \textsuperscript{b} (Bq \times \text{sec})</th>
<th>S-Factor (Gy/Bq \times \text{sec})</th>
<th>Dose to the cell nucleus (Gy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panitumumab-MCP-\textsuperscript{177}Lu</td>
<td>Panitumumab-MCP-\textsuperscript{177}Lu + \textsuperscript{177}Lu</td>
<td>Non-specific hIgG-MCP-\textsuperscript{177}Lu</td>
<td>Panitumumab-MCP-\textsuperscript{177}Lu</td>
</tr>
<tr>
<td>Cell surface</td>
<td>3989</td>
<td>367</td>
<td>544</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>2154</td>
<td>331</td>
<td>1040</td>
</tr>
<tr>
<td>Nucleus</td>
<td>663</td>
<td>101</td>
<td>468</td>
</tr>
<tr>
<td>Medium</td>
<td>9.8 \times 10^{10}</td>
<td>9.8 \times 10^{10}</td>
<td>9.8 \times 10^{11}</td>
</tr>
<tr>
<td>Total:</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Cells were incubated with 1.2 MBq (2.5 nmol/L) of radioimmunoconjugates for 24 h at 37 °C.

\textsuperscript{b} \(\bar{\text{A}}_s\): Cumulative radioactivity in the source compartment.

\textsuperscript{c} Cells were co-incubated with 1.2 MBq (2.5 nmol/L) of panitumumab-MCP-\textsuperscript{177}Lu and a 50-fold molar excess of unlabeled panitumumab to block EGFR.
3.4.3 Evaluation of normal tissue toxicity

Administration of 6.0 MBq (10 μg) of panitumumab-MCP-\textsuperscript{177}Lu to NRG mice caused no significant decrease in red blood cell (RBC), white blood cell (WBC) or platelet (PLT) counts (Figure 3-3a-c), Hb (Figure 3-3d) or HCT (Figure 3-3e) at 14 d post-injection compared to mice receiving normal saline. These results indicated no hematologic toxicity. In addition, there were no increases in serum ALT and only a slight increase in Cr in mice receiving RICs compared to normal saline treated mice (Figure 3-3f-g), indicating no liver or kidney toxicity.
Figure 3-3. Hematology and serum biochemistry analyses of NRG mice administered 6.0 MBq (10 μg) of panitumab-MCP-¹⁷⁷Lu or normal saline. (A) red blood cells [RBC], (B) white blood cells [WBC], (C) platelets [PLT], (D) hemoglobin [Hb], (E) hematocrit [HCT], (F) serum alanine aminotransferase [ALT], (G) serum creatinine [Cr]. Individual values are shown as well as the mean value (horizontal bars).
In addition, administration of panitumumab-MCP-$^{177}$Lu or non-specific hIgG-MCP-$^{177}$Lu caused no change in body weight over 14 d compared to mice treated with unlabeled panitumumab or normal saline, demonstrating no generalized normal tissue toxicity from these RICs administered at these amounts (Figure 3-4a).
Figure 3-4. (A) Body weight index (BWI) for tumour bearing NRG mice at different times post i.v. injection of 6.0 MBq (10 μg) of panitumumab-MCP-177Lu or non-specific hIgG-MCP-177Lu, unlabeled panitumumab (10 μg) or normal saline. Values shown are the mean ± SEM (n = 7) BWI. (B) Tumour growth index (TGI) in NRG mice with PANC-1 xenografts treated with a 6.0 MBq (10 μg) of panitumumab-MCP-177Lu or hIgG-MCP-177Lu or in mice receiving unlabeled panitumumab (10 μg) or normal saline. Values are the mean ± SEM (n = 7) TGI. Significant differences (P<0.05) between TGI at 33 days between treatment groups are indicated by asterisks.
3.4.4 Radioimmunotherapy studies

Administration of 6.0 MBq (10 μg) of panitumumab-MCP-\(^{177}\)Lu to NRG mice significantly inhibited the growth of PANC-1 xenografts (Figure 3-4b). The mean ± SEM TGI at 33 days in surviving NRG mice treated with panitumumab-MCP-\(^{177}\)Lu was 2.5 ± 0.3 (n=7) compared to 5.8 ± 0.5 for mice receiving normal saline (n=3; P<0.001). Treatment of NRG mice with non-specific hIgG-MCP-\(^{177}\)Lu (n=4) slightly but non-significantly inhibited tumour growth (TGI = 4.0 ± 0.7) compared to normal saline treated mice (P=0.11) but these RICs were less effective than panitumumab-MCP-\(^{177}\)Lu (P=0.04). Treatment of NRG mice with unlabeled panitumumab (10 μg; n=3) did not slow tumour growth compared to normal saline treated control mice (TGI = 6.1 ± 1.1; P=0.81).

3.4.5 Tumour and normal organ dosimetry

The absorbed doses in PANC-1 tumours and in normal organ doses in NRG mice were estimated based on the cumulative radioactivity (Ås) in the tumour or source organs (Table 3-2) without correction for radioactive decay determined in biodistribution studies (Figure 3-5 or 3-6) and using mouse organ S-values (323). Absorbed doses in NRG mice with s.c. PANC-1 tumours after i.v. injection of 6.0 MBq (10 μg) of panitumumab-MCP-\(^{177}\)Lu are shown in Table 3-3. The tumour dose for panitumumab-MCP-\(^{177}\)Lu was 12.3 ± 0.9 Gy while the highest normal organ doses were received by the pancreas (19.3 ± 6.5 Gy), kidneys (15.7 ± 3.7 Gy) and liver (7.5 ± 1.6 Gy). The whole body dose was 1.4 ± 0.7 Gy.
Figure 3.5. Radioactivity in selected source organs at 24, 72, 120 and 168 h post i.v. injection of 6.0 MBq (10 µg) of panitumumab-MCP-177Lu in NRG mice. The cumulative radioactivity in each source organ was calculated from the area-under-the-curve (AUC_{0-168h}; MBq × h). The AUC_{168-∞} was calculated by dividing the radioactivity at the last measured time point by the decay constant for 177Lu (0.004345 h^{-1}), assuming further elimination only by radioactive decay.
Figure 3-6. Tumour and normal organ biodistribution of panitumumab-MCP-\textsuperscript{177}Lu (MBq/g) in NRG mice with s.c. PANC-1 human pancreatic cancer xenografts at selected times up to 168 h post-injection. There was no correction for radioactive decay as these values were used for estimating the cumulative radioactivity in these source organs (A\textsubscript{s}) for radiation dosimetry calculations.
Table 3-2. Cumulative radioactivity (\(\bar{A}_S\)) in source organs for panitumumab-MCP-\(^{177}\)Lu injected i.v. in NRG mice with s.c. PANC-1 tumour xenografts.

<table>
<thead>
<tr>
<th>Source organ</th>
<th>(\bar{A}_S) from 0-168 h p.i. (MBq (\times) h) (^a)</th>
<th>(\bar{A}_S) from 168 to (\infty) (MBq (\times) h) (^b)</th>
<th>Total (\bar{A}_S) from 0 to (\infty) (MBq (\times) h) (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>7.39</td>
<td>3.72</td>
<td>11.11</td>
</tr>
<tr>
<td>Lungs</td>
<td>3.98</td>
<td>2.56</td>
<td>6.54</td>
</tr>
<tr>
<td>Liver</td>
<td>95.01</td>
<td>72.96</td>
<td>167.97</td>
</tr>
<tr>
<td>Spleen</td>
<td>15.59</td>
<td>25.13</td>
<td>40.72</td>
</tr>
<tr>
<td>Pancreas</td>
<td>8.14</td>
<td>13.90</td>
<td>22.04</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.81</td>
<td>4.16</td>
<td>4.97</td>
</tr>
<tr>
<td>Intestine</td>
<td>8.56</td>
<td>16.98</td>
<td>25.54</td>
</tr>
<tr>
<td>Kidneys</td>
<td>14.85</td>
<td>3.15</td>
<td>18.00</td>
</tr>
<tr>
<td>Tumour</td>
<td>23.89</td>
<td>29.58</td>
<td>53.47</td>
</tr>
</tbody>
</table>

\(^a\) Calculated by integrating the radioactivity vs. time curves for source organs shown in Figure 3-5.

\(^b\) Calculated by dividing the radioactivity at 168 h p.i. of panitumumab-MCP-\(^{177}\)Lu for source organs by the decay constant for \(^{177}\)Lu (0.004345 h\(^{-1}\)), assuming further elimination only by radioactive decay.

\(^c\) These values were converted to Bq \(\times\) sec for absorbed dose calculations (Table 3-3) by multiplying by \((3.6 \times 10^3\) secs/h)(\(10^6\) Bq/MBq).
Table 3-3. Estimated radiation absorbed doses in the tumour and normal organs in NRG mice with s.c. PANC-1 xenografts injected with panitumumab-MCP-^{177}Lu

<table>
<thead>
<tr>
<th>Target organ</th>
<th>Dose (Gy) (^a)</th>
<th>Major source organ contributor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>3.37 ± 0.39</td>
<td>Heart (94.9%)</td>
</tr>
<tr>
<td>Lungs</td>
<td>4.31 ± 0.54</td>
<td>Lungs (90.6%)</td>
</tr>
<tr>
<td>Liver</td>
<td>7.53 ± 1.55</td>
<td>Liver (98.8%)</td>
</tr>
<tr>
<td>Spleen</td>
<td>14.80 ± 2.76</td>
<td>Spleen (97.8%)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>19.31 ± 6.47</td>
<td>Pancreas (96.7%)</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.87 ± 0.38</td>
<td>Stomach (56.9%)</td>
</tr>
<tr>
<td>Intestine</td>
<td>3.22 ± 1.58</td>
<td>Intestine (87.1%)</td>
</tr>
<tr>
<td>Kidneys</td>
<td>15.66 ± 3.70</td>
<td>Kidneys (97.3%)</td>
</tr>
<tr>
<td>Whole Body</td>
<td>1.40 ± 0.69</td>
<td>Liver (10.6%)</td>
</tr>
<tr>
<td>Tumour (^b)</td>
<td>12.33 ± 0.86</td>
<td>Tumour (100%)</td>
</tr>
</tbody>
</table>

\(^a\) Dose (D) was estimated for i.v. injection of 6 MBq (10 \(\mu\)g) of panitumumab-MCP-^{177}Lu and represents the sum of all source organ→target organ pairs, calculated for each pair as was calculated as \(D = \bar{A}_s \times S\), where \(\bar{A}_s\) is the cumulative radioactivity in source organs (Table 3-2) and S are the Snyder values for mice (323).

\(^b\) Estimated using the sphere model in OLINDA/EXM (324) based on the measured tumour diameter.
3.5 Discussion

Polymers that present multiple chelators for complexing metal ions (MCP) conjugated to mAbs offer advantages compared to direct conjugation of chelators for radiometal labeling. For example, MCP-conjugated mAbs may be labeled to higher specific activity (SA). We previously reported that panitumumab modified with the same MCP described here presenting 13 DOTA chelators was labeled to a SA up to 72 MBq/µg with $^{177}$Lu, while achieving >90% labeling efficiency (LE), almost two-fold higher than panitumumab conjugated directly to two DOTA (46 MBq/µg) (312). Even greater differences (>10-fold) in SA were found between panitumumab-MCP and panitumumab-DOTA dual-labeled with $^{177}$Lu and $^{111}$In (257, 312). Higher SA provides greater potency in vitro for killing cancer cells, especially for cells that display low to moderate receptor expression (325). However, there are challenges in studying high SA RICs for RIT in vivo, since this requires administration of either a smaller mass or a larger radioactivity injected dose at the same mass. A small mass may cause high liver uptake (326), decreasing tumour uptake, while a larger radioactivity injected dose may exceed the maximum tolerated injected dose in mice. Therefore, in the current study, we administered a mass of RICs (10 µg) complexed to 6 MBq of $^{177}$Lu that we previously found localized well in s.c. PANC-1 tumours in NOD/SCID mice with only moderate liver uptake (312). Nonetheless, there remain practical advantages to MCP conjugation of mAbs, including the availability of multiple chelators to complex radiometals which minimizes interfering effects of contaminating trace metals or stable decay products often found in commercially available radiometal solutions (327). In our experience, MCP-conjugated mAbs provide very robust (LE>99%) and highly reliable (no failures encountered) radiometal complexation. These properties of MCP immunoconjugates would provide an advantage in formulating a kit for labeling a RIT agent, as high LE (>95%) and reliability are needed to minimize normal organ toxicities that may occur with unbound radionuclides present even at small amounts. Finally, a mAb may be modified at a single site with a MCP that presents multiple chelators, thus potentially avoiding decreased immunoreactivity due to derivatization of the mAb with multiple chelators at many different sites (328). Our studies described here provide to our knowledge the first report of RIT of tumours in mice with panitumumab conjugated to a MCP for complexing the β-emitter, $^{177}$Lu. These studies extend the theranostic application from our previous report (312) in which we
demonstrated that panitumumab-MCP-\(^{111}\)In imaged s.c. PANC-1 tumours in non-obese diabetic severe combined immunodeficiency (NOD/SCID) mice by microSPECT/CT.

Panitumumab was linked to two MCP by reaction of HyNIC-modified polymers with an S-4FB moiety introduced into the mAb (312). Panitumumab-MCP labeled with \(^{111}\)In exhibited high affinity EGFR binding (\(K_D = 2.2 \pm 0.6\) nmol/L) and was stable in vitro to loss of radiometal after challenge with EDTA or in human plasma (312). In the current study, we determined the cytotoxicity in vitro of panitumumab-MCP-\(^{177}\)Lu on PANC-1 cells by clonogenic survival (CS) assays and assessed lethal DNA DSBs inflicted by the emission of \(\beta\)-particles by \(^{177}\)Lu probing for \(\gamma\)-H2AX (315). Panitumumab-MCP-\(^{177}\)Lu decreased the CS of PANC-1 cells by 9.0-fold at the highest amount tested (1.2 MBq; Figure 3-1a) which was associated an increase in DNA DSBs (Figure 3-1b,c). The lowest activity of panitumumab-\(^{177}\)Lu tested (0.3 MBq) decreased the CS of PANC-1 cells by 1.4-fold (Figure 3-1a), but there was no increase in DNA DSBs at this amount compared to cells exposed to unlabeled panitumumab-MCP or untreated cells (Figure 3-1b,c). Nuclear DNA is believed to be the principal target for radiation induced lethal damage, but the cell membrane has also been shown to be an important target (213). Moreover, DNA DSBs are repairable, and it is possible that these were inflicted in PANC-1 cells but were repaired prior to probing for \(\gamma\)-H2AX at 16 h after exposure to this low amount of panitumumab-MCP-\(^{177}\)Lu. At higher amounts, the DNA repair processes are likely insufficient to repair all DNA DSBs. Unlabeled panitumumab-MCP exhibited no toxicity on PANC-1 cells, indicating that the polymer was not cytotoxic. Unlabeled panitumumab is not expected to be cytotoxic to PANC-1 cells, since these cells have a KRAS mutation that results in constitutive downstream EGFR activation not blocked by panitumumab (329).

Binding of panitumumab-MCP-\(^{177}\)Lu to PANC-1 cells was reduced by 11.8-fold in the presence of an excess of unlabeled panitumumab and the binding of non-specific hIgG-MCP-\(^{177}\)Lu to PANC-1 cells was 3.5-fold lower than panitumumab-MCP-\(^{177}\)Lu (Figure 3-2a). These results indicate EGFR-specific cell binding. The absorbed dose in the nucleus of PANC-1 cells by panitumumab-MCP-\(^{177}\)Lu was estimated by measuring the cumulative radioactivity (\(\bar{\alpha}\)) on the cell surface, in the cytoplasm or in the nucleus source compartments (Figure 3-2b and Table 3-1) and then applying microdosimetry models. A total of almost 4 Gy was deposited in the nucleus of PANC-1 cells at the highest amount of radioactivity studied (1.2 MBq; Table 3-1). The S-
value for cell-bound $^{177}$Lu in the cytoplasm ($4.02 \times 10^{-4}$ Gy/Bq × sec) was slightly greater than for $^{177}$Lu on the cell surface ($3.55 \times 10^{-4}$ Gy/Bq × sec) but the S-value was highest for $^{177}$Lu in the nucleus ($6.93 \times 10^{-4}$ Gy/Bq × sec). $^{177}$Lu emits moderate energy β-particles ($E_{\beta_{\text{max}}} = 0.18-0.5$ MeV) that have a maximum range of ~2 mm, which accounts for the comparable S-values for $^{177}$Lu on the cell surface or in the cytoplasm. However, there is a distribution of energies with many β-particles exhibiting lower energy than the $E_{\beta_{\text{max}}}$ (330). Low energy β-particles have a shorter range and would be more efficient for depositing dose in the nucleus when $^{177}$Lu is in the nucleus, than when it decays occur on the cell surface or in the cytoplasm. This cross-fire effect is expected due to the 2 mm range β-particles emitted by $^{177}$Lu.

For RIT studies, we confirmed that an administered amount of panitumumab-MCP-$^{177}$Lu (6.0 MBq; 10 µg) previously found to cause no major normal tissue toxicity for RIT using other $^{177}$Lu-labeled mAbs in NRG mice (318), would be safe to administer to NRG mice for RIT of PANC-1 tumours. NRG mice were selected for RIT because PANC-1 tumours efficiently engraft into these mice, but unlike NOD/SCID mice, these mice do not harbor a germ-line defect in DNA repair that renders NOD/SCID mice unusually radiation sensitive, including to RIT (319, 331). No decreases in CBC or increases in serum ALT or Cr compared to mice receiving normal saline were noted (Figure 3-3) indicating no hematopoietic, liver or kidney toxicity at these amounts. There was also no decrease in body weight in mice treated with panitumumab or with non-specific IgG RICs (Figure 3-4a), indicating no generalized normal tissue toxicity. These results are similar to previous studies by our group in which 3.7-11.1 MBq of $^{177}$Lu-labeled RICs bispecific for HER2 and EGFR were safely administered to non-tumour bearing Balb/c mice without normal tissue toxicity (332). Dose-limiting bone marrow toxicity has been reported at higher administered amounts in humans for $^{177}$Lu-labeled mAbs (333). In one study, the maximum tolerated dose (MTD) of $^{177}$Lu-labeled J591 for RIT of prostate cancer patients was 2,590 MBq/m², but two or three fractionated doses of 1,110-2220 MBq/m² were safely administered over 4-6 months (334). Assuming a body surface area of 0.009 m² for a mouse (334), the amount of panitumumab-MCP-$^{177}$Lu administered to NRG mice in our study was 667
MBq/m², which is lower than these amounts administered to humans. However, a challenge in assessing the toxicity of panitumumab-MCP-\(^{177}\)Lu in mice is that panitumumab does not bind the murine EGFR homologue, thus the absence of normal tissue toxicity from panitumumab-MCP-\(^{177}\)Lu in NRG mice may not preclude EGFR-mediated toxicity in humans. EGFR are present at low levels on most epithelial tissues but are moderately expressed by the liver and kidneys (335, 336). Importantly however is that <3% of hematopoietic stem cells in the bone marrow, which is one of the most radiation sensitive tissues are EGFR-positive (337). A Phase II trial of RIT with anti-EGFR mAb 425 labeled with the low energy, subcellular range Auger electron emitter, \(^{125}\)I improved survival in patients with glioblastoma multiforme without major (Grade 3 or 4) toxicities including to EGFR-positive tissues (e.g. bowel or skin) after administration of 3 weekly doses of 1,800 MBq (~26 MBq/kg) (338). Nonetheless, it remains possible that the more energetic and longer range β-particles emitted by \(^{177}\)Lu may cause toxicity, which would need to be evaluated in a Phase I clinical trial.

RIT with a single administered dose of panitumumab-MCP-\(^{177}\)Lu (6 MBq; 10 μg) strongly inhibited the growth of PANC-1 tumours in NRG mice for up to 26 days before tumour growth resumed (Figure 3-4b). In contrast, tumours in mice treated with unlabeled panitumumab or normal saline showed rapid exponential growth at all times. As mentioned, PANC-1 cells have a KRAS mutation which prevents panitumumab from inhibiting the growth of these tumours (329). hIgG-MCP-\(^{177}\)Lu also slowed the growth of PANC-1 tumours, but these non-specific RICs were significantly less effective than panitumumab-MCP-\(^{177}\)Lu. We did not study repeated treatment with panitumumab-MCP-\(^{177}\)Lu, but it is possible that tumour regrowth could be further inhibited by administering a second dose. In addition, it may be feasible to increase the administered dose to the maximum tolerated dose (MTD) to improve tumour response rather than the No Observable Adverse Effect Level (NOAEL) dose used in this study. Nonetheless, our results reveal that RIT with panitumumab-MCP-\(^{177}\)Lu was able to overcome KRAS mutation in PANC-1 tumours which caused resistance to panitumumab, suggesting that RIT could be a promising approach to treatment of PnCa in humans (308). Furthermore, treatment of small volume residual disease, including disseminated tumours to prevent metastatic progression after surgical resection of the primary tumour could be the future of pancreatic cancer treatment using RIT. We plan to conduct studies in an orthotopic patient derived xenograft (PDX) model of
pancreatic cancer that metastasizes to the liver to investigate the role of RIT in this context (339). We previously used this PDX model to study positron emission tomography (PET) of pancreatic cancer with $^{64}$Cu-DOTA-panitumumab F(ab')$_2$ fragments (143).

Finally, we estimated the absorbed doses in PANC-1 tumours and normal organs in mice using biodistribution data (Figure 3-5) from administration of panitumumab-MCP-$^{177}$Lu (Table 3-3). The tumour dose was high (12.3 Gy) in agreement with the strong tumour growth inhibition caused by panitumumab-MCP-$^{177}$Lu. The highest normal doses were received by the kidneys (15.7 Gy), pancreas (19.3 Gy), spleen (14.8 Gy) and liver (7.5 Gy). The MCP conjugated to panitumumab were PEGylated but liver and spleen uptake remains a challenge for polymer RICs. In other studies, we found that zwitterionic MCP conjugated to panitumumab provided lower liver and spleen uptake than polyanionic MCP used in the current study (270). Nonetheless, the absence of hepatic and renal toxicity (Figure 3-3) is consistent with the doses deposited in the liver and kidneys and the tolerance of these tissues to radiation, with doses $>$30 Gy most likely to cause with liver toxicity and $>$23-25 Gy to cause renal toxicity in humans (340). No bone marrow toxicity was found for panitumumab-MCP-$^{177}$Lu (Figure 3-3) despite a whole body absorbed dose of 1.4 Gy (Table 3-3). Whole body dose has been used as a predictor of bone marrow toxicity from RIT with doses $>$2-3 Gy causing dose-limiting hematopoietic toxicity in humans (333, 341). Mouse S-values were used to estimate the normal organ doses in mice from panitumumab-MCP-$^{177}$Lu (323). Due to the close proximity of organs in the mouse and the maximum 2 mm range of the $\beta$-particles emitted by $^{177}$Lu, the doses to normal organs in NRG mice may overestimate the human doses, due to a significant cross-fire effect. Bitar et al. point out that for energies $>$0.5 MeV, $\beta$-particles cannot be considered as non-penetrating radiation in mice, i.e. they have a significant cross-fire effect (323). This cross-fire effect is noted by the main contributing source organ for the dose to the whole body being the liver (Table 3-3).

3.6 Conclusion

We conclude that panitumumab-MCP-$^{177}$Lu inflicted DNA DSBs in PANC-1 human PnCa cells in vitro that decreased the CS of these cells. Administration of 6 MBq (10 $\mu$g) of panitumumab-MCP-$^{177}$Lu caused no normal tissue toxicity in NRG mice but strongly inhibited the growth of s.c. PANC-1 tumours for up to 26 days. Unlabeled panitumumab was not effective for treating
PANC-1 tumours due to KRAS mutation. Our results demonstrate that RIT with panitumumab-MCP-\textsuperscript{177}Lu was able to overcome resistance to panitumumab caused by KRAS mutation in PANC-1 tumours, suggesting that RIT may be a promising approach to the treatment of PnCa in humans.

3.7 Acknowledgements

This work was supported by a grant from the Canadian Cancer Society and a grant from the Cancer Research Society. S. Aghevlian received scholarships from the STARS21 strategic training program in radiation research supported by the Terry Fox Foundation and the Centre for Pharmaceutical Oncology (CPO) at the University of Toronto. S. Aghevlian is supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) through the Polymer Nanoparticles for Drug Delivery (POND) Training Program. The authors would like to thank Deborah Scollard at the Spatiotemporal Targeting and Amplification of Radiation Response (STTARR) Innovation Centre for technical support.
Chapter 4: Comparison of Auger Electron-Emitting $^{111}$In- or β-Particle-Emitting $^{177}$Lu Complexed to Panitumumab for Radioimmunotherapy of EGFR-Positive PANC-1 Human Pancreatic Cancer Xenografts in NOD/SCID and NRG Mice
This chapter will be submitted to Molecular Pharmaceutics. All experiments were designed and carried out by Sadaf Aghevlian. Dosimetry was performed with assistance from Dr. Zhongli Cai. The synthesis and characterization of metal chelating polymer were performed by Dr. Yijie Lu at the Department of Chemistry at the University of Toronto.
4. Abstract

Our hypothesis was that panitumumab modified with two DOTA chelators or with metal-chelating polymers (MCP) that present 13 DOTA chelators to complex the β-particle emitter, $^{177}$Lu or Auger electron (AE)-emitter, $^{111}$In, would be effective for radioimmunotherapy (RIT) of subcutaneous (s.c.) EGFR-positive PANC-1 human PnCa xenografts in mice at administered amounts that are non-toxic to normal tissues. The clonogenic survival (CS) of PANC-1 cells exposed in vitro to panitumumab-DOTA-$^{177}$Lu, panitumumab-DOTA-$^{111}$In or panitumumab-MCP-$^{111}$In was determined. DNA double-strand breaks (DSBs) were assessed by immunofluorescence for γ-H2AX. Absorbed doses to the cell nucleus was calculated based on subcellular distribution of the radioimmunoconjugates (RICs). Normal tissue toxicity in vivo was assessed by monitoring body weight, complete blood cell (CBC) counts and serum alanine aminotransferase (ALT) and creatinine (Cr) in NOD/SCID mice injected i.v. with 10 MBq (10 μg) of panitumumab-MCP-$^{111}$In or panitumumab-DOTA-$^{111}$In, or in NRG mice administered 6 MBq (10 μg) of panitumumab-DOTA-$^{177}$Lu. For RIT, NOD/SCID mice or NRG mice with s.c. PANC-1 tumours were administered three amounts (10 MBq; 10 μg) of panitumumab-MCP-$^{111}$In or panitumumab-DOTA-$^{111}$In separated by 3 weeks or a single amount (6 MBq; 10 μg) of panitumumab-DOTA-$^{177}$Lu, respectively. Control mice were treated with normal saline or unlabeled panitumumab. Tumour growth was assessed by a tumour growth index (TGI). Biodistribution studies were performed to calculate the cumulative radioactivity ($\bar{A}_s$) in normal organs and tumour. The absorbed doses were estimated using $D = \bar{A}_s \times S$ equation and mouse S-values. The tumour dose was estimated using OLINDA/EXM and the sphere model. Panitumumab-DOTA-$^{177}$Lu was 5-fold and 6.4-fold more cytotoxic than panitumumab-MCP-$^{111}$In or panitumumab-DOTA-$^{111}$In, respectively, against PANC-1 cells which was associated with more DNA DSBs and higher absorbed doses to the cell nucleus. There were no differences in the cytotoxicity in vitro of panitumumab-MCP-$^{111}$In and panitumumab-DOTA-$^{111}$In. None of the RICs caused any significant changes in body weight, decreased CBC or increases in ALT or Cr compared to normal saline treated mice. Panitumumab-MCP-$^{111}$In or panitumumab-DOTA-$^{111}$In inhibited PANC-1 tumour growth in NOD/SCID mice (TGI at 43 days $= 3.9 \pm 0.3$ and $3.0 \pm 0.4$, respectively; $P > 0.05$) compared to normal saline and panitumumab treated mice (TGI $= 9.8 \pm 1.6$, $9.9 \pm 1.4$, respectively; $P < 0.001$). Similarly, panitumumab-DOTA-$^{177}$Lu inhibited tumour...
growth in NRG mice (TGI = 2.9 ± 0.4). Absorbed doses in PANC-1 tumours were 11.5 ± 4.9 Gy for panitumumab-DOTA-$^{177}$Lu and 2.8 ± 0.7 Gy and 6.0 ± 2.2 Gy for panitumumab-MCP-$^{111}$In and panitumumab-DOTA-$^{111}$In; respectively (P>0.05). The spleen and liver uptake were higher for the MCP conjugates which makes these less attractive for RIT with $^{111}$In than the DOTA conjugates. Generally, lower absorbed doses in normal organs for $^{111}$In than $^{177}$Lu may suggest further escalation of the administered amount of $^{111}$In, while the whole body dose for $^{177}$Lu doesn’t allow further escalation in the administered amount because of potential bone marrow toxicity.
4.1 Introduction

In recent years there has been an increasing interest in targeting cancers overexpressing epidermal growth factor receptor (EGFR) using the fully humanized anti-EGFR monoclonal antibody (mAb), panitumumab (Vectibix, Amgen) (143, 286, 314, 342). Although pancreatic cancer (PnCa) (274) overexpresses EGFR, it has the highest incidence of KRAS mutation among all the EGFR positive cancers (309, 310) and cannot benefit from immunotherapy with panitumumab due to constitutive KRAS-mediated downstream signal activation (311). In this case, radioimmunotherapy (RIT) which exploits panitumumab only as a carrier for delivery of radioisotopes to tumours could be the best treatment approach. We recently proposed a novel metal chelating polymer (MCP) composed of a polyglutamide backbone and 13 DOTA (1,4,7,10-tetraazaacyclododecane-1,4,7,10-tetraacetic acid) chelators for radiolabeling panitumumab at high specific activity (SA) and presenting 10 pendant polyethylene glycol (PEG) groups in order to reduce spleen and liver uptake of the radioimmunoconjugates (RICs) (257). We showed that single intravenous (i.v.) injection (6 MBq; 10 μg) of panitumumab-MCP labeled with $^{177}$Lu [β particles; Eβ(max)=498 keV (78.6%), Eβ(max)=385 keV (9.1%) and Eβ(max)=176 keV (12.2%); t1/2 = 6.7 days], delayed the tumour growth of subcutaneous (s.c.) PANC-1 human PnCa xenografts in NOD Rag1−/−IL2RgammaC-null (NRG) mice by about 2.6-fold compared to control mice treated with normal saline or unlabeled panitumumab (343). In a separate study, we showed that panitumumab-MCP and panitumumab-DOTA labeled with the γ-emitter $^{111}$In [Eγ = 171 keV (90%) and Eγ = 245 keV (94%); (t1/2 = 2.8 d)] were able to image s.c. PANC-1 xenografts in nonobese diabetic severe combined immunodeficiency (NOD-SCID) mice at 72 h post injection (p.i.) by single photon emission computed tomography (SPECT) with a tumour uptake of 6.9 ± 1.3 % injected dose per gram (%ID/g) and 6.6 ± 3.3 %ID/g; respectively (257). $^{111}$In also emits 15 low energy (<25 keV) and nanometer-micrometer range Auger electrons (AEs) that are well suited for eradicating micrometastases since the radiation dose is deposited within the target volume of a single cell (200). The subcellular range of these electrons yields high linear energy transfer (LET = 4-26 keV) that causes lethal DNA double-strand breaks (DSBs), particularly if these electrons are emitted near the cell nucleus (191). RIT with $^{111}$In-labeled mAbs has proven effective for treatment of tumour xenografts for a range of cancers in mice (332, 344, 345). The potency of AEs can be enhanced by modification of the mAbs with nuclear translocation sequences (NLS) to promote nuclear uptake after receptor-
mediated internalization (346, 347). However, since the EGFR harbours a putative NLS in the transmembrane domain (348) that transports EGF (349) and anti-EGFR mAbs (350) to the nucleus of cancer cells following receptor mediated internalization, there is no need to modify panitumumab with NLS peptides to enhance the effectiveness of the AEs emitted by $^{111}$In (286, 343).

In the current study, we compared the 2 mm range β-particles emitted by $^{177}$Lu and the nanometer range AEs emitted by $^{111}$In for killing PANC-1 pancreatic cancer cells in vitro, and for treatment of PANC-1 xenografts in vivo in NRG mice and NOD/SCID mice. We also now report the therapeutic efficacy of panitumumab-MCP-$^{111}$In for comparison with our previously reported results using panitumumab-MCP-$^{177}$Lu (343). Our aim was to understand whether or not the β-particles emitted by $^{177}$Lu or AEs emitted by $^{111}$In would be the most effective for RIT of PANC-1 tumours in mice at administered amounts that were non-toxic to normal tissues. We compared the normal tissue toxicity of panitumumab-DOTA-$^{111}$In, panitumumab-DOTA-$^{177}$Lu and panitumumab-MCP-$^{111}$In.

4.2 Materials and methods

4.2.1 Cell culture and tumour xenografts

EGFR positive PANC-1 human pancreatic adenocarcinoma cells (4.0 × $10^5$ EGFR/ cell) (313) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (Gibco Life Technologies; Burlington, ON, Canada) supplemented with 1% penicillin and streptomycin and 10% fetal bovine serum (Gibco-Invitrogen). Tumour xenografts (7-9 mm diameter) were established in female NOD/SCID mice (Charles River) or in NRG mice (University Health Network) by s.c. inoculation of 3-4 × $10^6$ PANC-1 cells in serum free DMEM (100 μL) into the left flank. All animal studies were conducted in compliance with Canadian Council on Animal Care (CCAC) guidelines and were carried out under a protocol approved by the Animal Care Committee at the University Health Network (AUP 2843.3).
4.2.2 Radioimmunoconjugates (RICs)

The synthesis of hydrazino nicotinamide (HyNic) end-capped MCPs (HyNic-MCP) presenting 13 DOTA chelators and 10 PEG chains was previously reported (271). Panitumumab-MCP immunoconjugates were constructed by cross-linking panitumumab IgG to on average 2 HyNic-MCPs through an N-succinimidyl-4-formylbenzamide (S-4FB) moiety introduced into panitumumab (257). Panitumumab or non-specific IgG (hIgG; Sigma-Aldrich, St. Louis, MO, Product No. 14506) was also modified with on average 2 N-hydroxysuccinimide ester (DOTA-NHS; Macrocyclics) to construct panitumumab-DOTA conjugates (257). Unconjugated MCPs or DOTA-NHS were removed from panitumumab-MCP or panitumumab-DOTA and buffer-exchanged for subsequent radiolabeling by diluting in 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer pH 5.0, and passing through an Amicon spin filter (0.5 mL; 30 kDa MWC; Millipore). Purified immunoconjugates (5–70 μg; 4–12 μL) were then labeled with (0.75–49 MBq) $^{111}$InCl$_3$ (Nordion) or $^{177}$LuCl$_3$ (PerkinElmer) at 42 °C for 2 h (257).

The final radiochemical purity of RICs was >95% evaluated by instant thin-layer silica gel chromatography (ITLC-SG; Pall) developed in 0.1M sodium citrate, pH 5.5. The $K_d$ for binding of panitumumab-MCP-$^{177}$Lu and panitumumab-DOTA-$^{177}$Lu to EGFR was 2.2 ± 0.6 nmol/L and 1.0 ± 0.4 nmol/L; respectively (257). The $K_d$ values of the $^{111}$In-labeled RICs was not measured as it was assumed that these would not be changed by complexation of a different radiometal.

4.2.3 Clonogenic survival (CS)

The cytotoxicity of panitumumab-DOTA-$^{177}$Lu, panitumumab-DOTA-$^{111}$In and panitumumab-MCP-$^{111}$In on PANC-1 cells were compared in clonogenic survival (CS) assays. Briefly, 2 × 10$^5$ cells were treated with growth medium containing 2.5 nmoles/L (50-fold molar excess) of RICs: 0.3, 0.6 or 1.2 MBq [specific activity (SA) = 46.7, 93.5, 187.0 MBq/nmole] in 24 well plates for 16 h. Controls consisted of cells exposed to unlabeled panitumumab-DOTA or growth medium alone. Approximately 700 cells were then seeded into 6 well plates and cultured for 12 d. Surviving colonies were stained with methylene blue and colonies of more than 50 cells were counted. The plating efficiency (PE) was determined by dividing the number of colonies formed by the number of cells seeded. The CS was calculated by dividing the PE for treated cells by that for untreated cells.
4.2.4 Assessment of DNA damage

The accumulation of phosphorylated histone-2A (γ-H2AX) in the nucleus of PANC-1 cells due to DNA double-stranded breaks (DSBs) caused by panitumumab-DOTA-\(^{177}\)Lu, panitumumab-DOTA-\(^{111}\)In and panitumumab-MCP-\(^{111}\)In was assessed by immunofluorescence confocal microscopy (315). Briefly, \(2 \times 10^5\) cells were seeded onto glass coverslips (Ted Pella) in 24-well plates and cultured overnight at 37 °C. The cells were then incubated with the RICs or unlabeled panitumumab-DOTA or growth medium as described earlier. Immunostaining for γ-H2AX and analysis of the images for the density of foci per nucleus area was conducted according to previously reported procedures (316).

4.2.5 Subcellular distribution and cellular dosimetry

The subcellular distribution of panitumumab-DOTA-\(^{177}\)Lu in PANC-1 cells was measured as previously described for panitumumab-MCP-\(^{177}\)Lu and non-specific hIgG-MCP-\(^{177}\)Lu (343). Briefly, \(2 \times 10^5\) cells were cultured overnight in wells in a 6-well plate. Cells were then incubated with a 50-fold molar excess of panitumumab-DOTA-\(^{177}\)Lu (1.2 MBq; 2.5 nmoles/L) in the absence or presence of excess unlabeled panitumumab (EGFR blocking; control group) or with non-specific IgG-DOTA-\(^{177}\)Lu (control group) for different times (1, 4, 8, and 24 h) at 37 °C. The medium was removed and the cells rinsed with phosphate-buffered saline (PBS), pH 7.5. Cell-surface radioactivity was displaced with 1 mL of 200 mM sodium acetate/500 mM NaCl, pH 2.5 at 22 °C. This procedure was repeated twice and the combined cell membrane fractions measured in a γ-counter. In order to determine the proportion of cell-bound radioactivity internalized in the nucleus or remaining in cytoplasm, the cell membrane was lysed twice in 500 μL of Nuclei EZ Lysis buffer (Sigma-Aldrich) on ice for 60 min. The cells were gently detached from each well using a cell scraper and were transferred to 1.5 mL Eppendorf tubes. The tubes were centrifuged at 3,000 \(\times\) g for 5 min to separate nuclear and cytoplasmic radioactivity (supernatant). The fractions of nuclear and cytoplasmic radioactivity were measured separately in a γ-counter and the percentage of radioactivity in each fraction (relative to total cell-bound \(^{177}\)Lu) was calculated.
The absorbed dose in the nucleus of a PANC-1 cell was determined as previously described for panitumumab-MCP-\textsuperscript{177}Lu and non-specific hIgG-MCP-\textsuperscript{177}Lu (343). Briefly, the cumulative amount of radioactivity (\(\tilde{\text{A}}\text{s}\)) in the nucleus (D) after exposure to 1.2 MBq of panitumumab-DOTA-\textsuperscript{177}Lu (2.5 nmole/L) was estimated by plotting the amount of radioactivity in the nucleus vs. time and determining the area under the curve (\(\text{AUC}_{0-24h} \times \text{Bq} \times \text{sec}\)) using Prism Ver. 4.0 software (GraphPad). The absorbed dose in the nucleus was then estimated as \(D = \tilde{\text{A}}\text{s} \times S\), and the S-values (Gy/Bq \times sec) were calculated using MCNP Monte Carlo code Ver. 5 (Los Alamos National Laboratory). Following staining of the nucleus with 4',6-diamidino-2-phenylindole (DAPI), the mean cell radius and nucleus radius of PANC-1 cells were determined (17.1 \(\mu\text{m}\) and 7.0 \(\mu\text{m}\); respectively) by fluorescence microscopy of cells and ImageJ software (National Institutes of Health, Bethesda, MD) (221). The absorbed doses in the nucleus were also calculated for the control groups. The absorbed doses in the nucleus from the corresponding panitumumab-DOTA-\textsuperscript{111}In and hIgG-DOTA-\textsuperscript{111}In as well as panitumumab-MCP-\textsuperscript{111}In and hIgG-MCP-\textsuperscript{111}In were estimated assuming that these would exhibit similar subcellular distribution as panitumumab-DOTA-\textsuperscript{177}Lu and panitumumab-MCP-\textsuperscript{177}Lu (343). The \(t_{1/2}\) of \textsuperscript{111}In (2.8 d) was employed to estimate \(\tilde{\text{A}}\text{s}\), and the S-factors for \textsuperscript{111}In were used to calculate these radiation doses (323).

4.2.6 Evaluation of normal tissue toxicity

To evaluate normal tissue toxicity, groups of 5 healthy female NOD/SCID or NRG mice were injected i.v. with an amount of panitumumab-DOTA-\textsuperscript{111}In or panitumumab-MCP-\textsuperscript{111}In (10.0 MBq; 10 \(\mu\text{g}\)) previously reported to be safe in other preclinical RIT studies (351). Similarly, an amount of panitumumab-DOTA-\textsuperscript{177}Lu (6.0 MBq; 10 \(\mu\text{g}\)) previously found to be safe was studied (318, 343). NOD/SCID mice harbor a germ line mutation in DNA repair (319) which renders these mice unusually sensitive to radiation. Thus the toxicity of panitumumab-DOTA-\textsuperscript{177}Lu was examined in NRG mice which do not have this mutation since it was anticipated that the \(\beta\)-particles emitted by \textsuperscript{177}Lu may be more toxic to normal tissues than the AEs emitted by \textsuperscript{111}In, due to an associated cross-fire effect (191). Control mice received injections of normal saline or unlabeled panitumumab (10 \(\mu\text{g}\)). Body weight was monitored every 2-4 d for 14 d. The mice were then sacrificed and samples of blood collected into EDTA-coated microtubes for biochemistry [serum alanine aminotransferase (ALT) and creatinine (Cr)] and hematology.
analyses. A complete blood cell (CBC) count, hematocrit (HCT) and hemoglobin (Hb) were measured on a HemaVet 950FS (Drew Scientific) instrument.

4.2.7 Radioimmunotherapy (RIT) studies

The tumour-growth inhibitory effects of the RICs were studied in mice engrafted s.c. with PANC-1 xenografts. NOD/SCID mice received three i.v. injections of 10 MBq of panitumumab-DOTA-\(^{111}\)In, panitumumab-MCP-\(^{111}\)In or hIgG-MCP-\(^{111}\)In (10μg/100 μL) every three weeks whereas due to the longer half-life of \(^{177}\)Lu NRG mice received a single injection of 6 MBq of panitumumab-DOTA-\(^{177}\)Lu (10μg/100μL). Tumour length (mm) and width (mm) were measured using calipers every 2-3 days until a humane endpoint of the animal care protocol was reached. The tumour volume (V=mm\(^3\)) was calculated as V=length×width\(^2\)×0.5 (321). The tumour growth index (TGI) was calculated by dividing the tumour volume at each time point by the tumour volume at the start of treatment. Similarly, the body weight index (BWI) was estimated by dividing the body weight at each time point by the pre-treatment body weight. The results were compared to the control groups received saline (100μL) or unlabeled panitumumab (10μg/100μL).

4.2.8 Tumour and normal organ dosimetry

The absorbed doses in the tumour and normal organs in NOD/SCID or NRG mice with s.c. PANC-1 xenografts after injection of three amounts of \(^{111}\)In-labeled panitumumab-DOTA or panitumumab-MCP (10 MBq; 10 μg) or one amount of panitumumab-DOTA-\(^{177}\)Lu (6 MBq; 10 μg) were estimated as described previously (343). Briefly, groups of 5 NOD/SCID or NRG mice were injected i.v. with one amount of these RICs and at selected time points from 24 to 168 h p.i., the mice were sacrificed and the tumour and samples of normal organs collected and the radioactivity measured in a \(\gamma\)-counter. The cumulative radioactivity in the tumour and these normal source organs from 0-168 h \(\tilde{A}_{0-168h}\) was estimated from the area under the curve \([\text{AUC}_{0-168h}; \text{Bq} \times \text{sec}]\) using Prism Ver. 4.0 software (GraphPad). The cumulative radioactivity from 168 h to infinity \(\tilde{A}_{168h-\infty}\) was calculated by assuming elimination by physical decay without further biological clearance. By multiplying the combined \(\tilde{A}_{0-\infty}\) values by the S-value (Gy/Bq × sec) for \(^{111}\)In or \(^{177}\)Lu using published S-values for a mouse (323), the absorbed dose (Gy) for each organ
was estimated. The dose delivered to the tumour was estimated based on the S-values using the sphere model in OLINDA/EXM radiation dose software, and the measured tumour size (324). The absorbed dose for $^{111}$In-labeled RICs were estimated for three injections.

4.3 Statistical analysis

Results were expressed as mean ± SEM. Statistical significance was tested using an unpaired two-tailed Student’s t-test or one-way ANOVA (p<0.05).

4.4 Results

4.4.1 Clonogenic survival (CS)

There was no effect of unlabeled panitumumab-DOTA on the CS of PANC-1 cells (Figure. 4-1). There were no significant differences in the CS of PANC-1 cells exposed to panitumumab-MCP-$^{111}$In or panitumumab-DOTA-$^{111}$In at 0.3 MBq (67.92 ± 4.35 vs 83.01 ± 5.44; P>0.5), 0.6 MBq (64.91 ± 5.75 vs 71.48 ± 4.01) or 1.2 MBq (53.85 ± 6.85 vs 68.86 ± 3.34; P>0.5). However, at all radioactivity amounts, $^{177}$Lu was more cytotoxic than $^{111}$In. At the highest amount of radioactivity tested (1.2 MBq) panitumumab-DOTA-$^{177}$Lu (10.69 ± 2.45) was 5-fold and 6.4-fold more potent at reducing the CS of PANC-1 cells than panitumumab-MCP-$^{111}$In or panitumumab-DOTA-$^{111}$In, respectively.
Figure 4-1. Clonogenic survival of PANC-1 cells exposed to increasing radioactivity (0.3 MBq, 0.6 MBq or 1.2 MBq) of panitumumab-DOTA-\textsuperscript{177}Lu, panitumumab-MCP-\textsuperscript{111}In or panitumumab-DOTA-\textsuperscript{111}In (2.5 nmoles/L) or to unlabeled panitumumab-DOTA (2.5 nmoles/L; 0.0 MBq) for 16 h then cultured for 12 d. Data shown are the mean ± SEM (n = 3–4). Significant differences (P<0.05) between \textsuperscript{111}In- and \textsuperscript{177}Lu- labeled RICs are indicated by the asterisks. There are no significant differences between panitumumab-MCP-\textsuperscript{111}In and panitumumab-DOTA-\textsuperscript{111}In.
4.4.2 Assessment of DNA damage

There were no differences in the integrated density of $\gamma$-H2AX foci per nucleus area in the nucleus of cells exposed to growth medium and unlabeled panitumumab-DOTA ($0.69 \pm 0.05\%$ vs $0.76 \pm 0.10\%$; $P > 0.05$) (Figure 4-2A,B). At all radioactivity amounts (0.3, 0.6 and 1.2 MBq; 2.5 nmoles/L), panitumumab-DOTA-$^{177}$Lu caused more DNA DSBs than panitumumab-MCP-$^{111}$In or panitumumab-DOTA-$^{111}$In. At 1.2 MBq, panitumumab-DOTA-$^{177}$Lu caused 2.7-fold and 1.6-fold higher $\gamma$-H2AX foci than $^{111}$In-labeled panitumumab-DOTA or panitumumab-MCP ($3.1 \pm 0.3\%$ vs $1.1 \pm 0.2\%$ and $2.0 \pm 0.2\%$). Exposure of cells to increasing amounts of panitumumab-DOTA-$^{177}$Lu increased $\gamma$-H2AX foci. Panitumumab-DOTA-$^{111}$In at 1.2 MBq caused more DNA DSBs than at 0.3 MBq but not at 0.6 MBq (Figure 4-2B).
Figure 4-2. (A) Representative confocal immunofluorescence microscopy images assessing $\gamma$-H2AX foci (green foci) representing sites of unrepaired DNA DSBs in PANC-1 cells exposed for 16 h to 0.3 MBq, 0.6 MBq or 1.2 MBq of $^{177}$Lu-labeled panitumumab-DOTA or $^{111}$In-labeled panitumumab-DOTA or panitumumab-MCP. Cell nuclei were counterstained with DAPI (blue). In the bottom set of panels are images of PANC-1 cells exposed to growth medium alone, or unlabeled panitumumab-DOTA. (B) $\gamma$-H2AX foci were quantified as the integrated density/nucleus area and are shown for untreated PANC-1 cells, cells treated with unlabeled panitumumab-DOTA, panitumumab-MCP (343) or increasing amounts of RICs. Values shown represent the mean ± SEM (n=3-4). Significant differences (P<0.05) between the RICs are indicated by the asterisks.
4.4.3 Subcellular distribution and cellular dosimetry

The percentage of radioactivity bound to PANC-1 cells increased over time for all RICs. The percentage of cell-bound radioactivity for panitumumab-DOTA-\(^{177}\)Lu (11.0 ± 0.2%) was significantly higher at 24 h after incubation compared to that for non-specific hIgG-DOTA-\(^{177}\)Lu (0.50 ± 0.32%; P<0.001) or panitumumab-DOTA-\(^{177}\)Lu in the presence of 50-fold molar excess unlabeled panitumumab (0.80 ± 0.05%; P< 0.001) indicating the EGFR cell-binding specificity of the panitumumab-DOTA conjugates (Figure 4-3A). There were no significant changes with time in the proportion of cell-bound radioactivity on the membrane, in the cytoplasm or nucleus for panitumumab-DOTA-\(^{177}\)Lu (Figure 4-3B).
Figure 4-3. (A) Percentage of cell-bound radioactivity at selected time points (1, 4, 8 or 24 h) after incubation of PANC-1 cells with panitumumab-DOTA-\textsuperscript{177}Lu with or without excess unlabeled panitumumab or with hIgG-DOTA-\textsuperscript{177}Lu. (B) Percentage of radioactivity on the membrane, in the cytoplasm or nucleus of PANC-1 cells at selected times after incubation with panitumumab-DOTA-\textsuperscript{177}Lu.
The subcellular fractionation data for all the time points were used to estimate absorbed doses in the nucleus of PANC-1 cell from $^{111}$In- or $^{177}$Lu-labeled panitumumab-DOTA assuming that substitution of $^{111}$In for $^{177}$Lu would not change cell binding or subcellular localization (Table 4-1 and 4-2). Similarly, the absorbed dose in the nucleus from panitumumab-MCP-$^{111}$In was calculated based on the subcellular distribution of panitumumab-MCP-$^{177}$Lu which has been published in our previous study (343). The dose deposited in the nucleus was 3.5-fold and 6.9–fold higher for panitumumab-DOTA-$^{177}$Lu than panitumumab-DOTA-$^{111}$In and panitumumab-MCP-$^{111}$In, respectively (Table 4-1 and 4-2). For the cells incubated with radiolabeled hIgG-DOTA or hIgG-MCP or co-incubated with excess unlabeled panitumumab the absorbed doses to the nucleus were significantly lower and radioactivity in the surrounding growth medium was the major source contributor to the dose (Table 4-1 and 4-2).
Table 4-1. Cumulative radioactivity in the source component ($\bar{A}_s(0-\infty)$) based on subcellular distribution of radioactivity after incubation of PANC-1 cells with 1.2 MBq of $^{111}$In-labeled RICs (2.5 nmoles/L) in the absence or presence of 50-fold excess unlabeled panitumumab for 24 h at 37 °C.

<table>
<thead>
<tr>
<th>Source compartment</th>
<th>S (Gy/Bq×sec) $^{111}$In</th>
<th>Panitumumab-DOTA</th>
<th>Panitumumab-DOTA+ excess panitumumab</th>
<th>hIgG-DOTA</th>
<th>Panitumumab-MCP*</th>
<th>Panitumumab-MCP + excess panitumumab</th>
<th>hIgG-MCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell surface</td>
<td>$7.35 \times 10^{-5}$</td>
<td>1.01</td>
<td>0.06</td>
<td>0.02</td>
<td>0.29</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>$1.03 \times 10^{-4}$</td>
<td>0.3</td>
<td>0.01</td>
<td>0.007</td>
<td>0.22</td>
<td>0.03</td>
<td>0.1</td>
</tr>
<tr>
<td>Nucleus</td>
<td>$5.06 \times 10^{-4}$</td>
<td>0.64</td>
<td>0.04</td>
<td>0.06</td>
<td>0.34</td>
<td>0.05</td>
<td>0.23</td>
</tr>
<tr>
<td>Medium</td>
<td>$3.39 \times 10^{-12}$</td>
<td>0.33</td>
<td>0.33</td>
<td>0.33</td>
<td>0.32</td>
<td>0.33</td>
<td>0.33</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>2.28</td>
<td>0.44</td>
<td>0.42</td>
<td>1.17</td>
<td>0.43</td>
<td>0.69</td>
</tr>
</tbody>
</table>

Major source contributor (%): Membrane (44.3%), Medium (75.0%), Medium (79%), Nucleus (29%), Medium (76.7%), Medium (47.8%).

*The subcellular distribution of $^{177}$Lu–labeled RICs was previously reported and used to estimate the dose from the corresponding $^{111}$In-labeled RICs.
Table 4-2. Cumulative radioactivity in the source component ($\bar{A}_{s(0-\infty)}$) based on subcellular distribution of radioactivity after incubation of PANC-1 cells with 1.2 MBq of panitumumab-DOTA-$^{177}$Lu (2.5 nmoles/L) in the absence or presence of 50-fold excess unlabeled panitumumab for 24 h at 37 °C.

<table>
<thead>
<tr>
<th>Source compartment</th>
<th>Dose to the cell nucleus (Gy)</th>
<th>Panitumumab-DOTA</th>
<th>Panitumumab-DOTA+ excess panitumumab</th>
<th>hIgG-DOTA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S (Gy/Bq×sec) Lu-177</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell surface</td>
<td>$3.55 \times 10^{-4}$</td>
<td>4.86</td>
<td>0.3</td>
<td>0.12</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>$4.02 \times 10^{-4}$</td>
<td>1.18</td>
<td>0.05</td>
<td>0.03</td>
</tr>
<tr>
<td>Nucleus</td>
<td>$6.93 \times 10^{-4}$</td>
<td>0.87</td>
<td>0.05</td>
<td>0.09</td>
</tr>
<tr>
<td>Medium</td>
<td>$1.15 \times 10^{-11}$</td>
<td>1.13</td>
<td>1.13</td>
<td>1.13</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>8.06</strong></td>
<td><strong>1.53</strong></td>
<td><strong>1.36</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Major source contributor (%)</strong></td>
<td>Membrane (60.3%)</td>
<td>Medium (73.9%)</td>
<td>Medium (83.1%)</td>
<td></td>
</tr>
</tbody>
</table>
4.4.4 Evaluation of normal tissue toxicity

Administration of 10.0 MBq (10 µg) of panitumumab-DOTA-\(^{111}\)In and panitumumab-MCP-\(^{111}\)In to non-engrafted NOD/SCID mice or 6.0 MBq (10 µg) of panitumumab-MCP-\(^{177}\)Lu to NRG mice caused no decrease in white blood cell (WBC), red blood cell (RBC) counts or hemoglobin (Hb) (Figure 4A-C) or in hematocrit (HCT) (Figure 4D) or platelet (PLT) counts (Figure 4E) at 14 d compared to NOD/SCID mice receiving normal saline. In addition, there were no increases in serum ALT or Cr in mice receiving panitumumab-DOTA-\(^{111}\)In compared to normal saline treated mice (Figure 4F,G). Similarly, there was no significant difference in the blood analysis of the NRG mice administered 6.0 MBq (10 µg) of panitumumab-DOTA-\(^{177}\)Lu compared to the control NRG mice receiving normal saline injection which was reported in our previous study (343). There were some mouse strain differences noted between NOD/SCID mice or NRG mice in serum ALT or Cr in normal saline controls (343). No significant differences were observed in the body weight between the control group and treatment groups.
Figure 4-4. Hematology and serum biochemistry analyses of NOD/SCID mice at 14 d after i.v. injection of 10.0 MBq (10 µg) of panitumumab-DOTA-\(^{111}\)In, panitumumab-MCP-\(^{111}\)In or normal saline. Also shown is the data for NRG mice administered 6.0 MBq (10 µg) of panitumumab-DOTA-\(^{177}\)Lu or normal saline (taken from the previous study (343). (A) white blood cells [WBC], (B) Red blood cells [RBC], (C) hemoglobin [Hb], (D) hematocrit [HCT], (E) platelets [PLT], (F) serum creatinine [Cr], (G) serum alanine aminotransferase [ALT]. Individual values are shown as well as the mean (horizontal bars). No significant difference was observed between normal saline control and treatment groups.
4.4.5 Radioimmunotherapy (RIT) studies

Administration of three single amounts (10.0 MBq; 10 μg) of panitumumab-DOTA-\(^{111}\)In or panitumumab-MCP-\(^{111}\)In separated by 3 weeks to NOD/SCID mice strongly inhibited PANC-1 tumour growth (Figure 4-5A). There was no significant difference in the mean TGI at 43 d (time point selected to include >3 surviving mice per group) for mice treated with panitumumab-DOTA-\(^{111}\)In or panitumumab-MCP-\(^{111}\)In (3.97 ± 0.3 vs. 3.0 ± 0.4, respectively; P>0.05) but the TGI for treatment with these \(^{111}\)In-labeled RICs was 2.5-3.0 fold significantly lower than for control mice receiving normal saline or unlabeled panitumumab (TGI = 9.8 ± 1.6 and 9.9 ± 1.4, respectively; P<0.001). Administration of a single amount (6.0 MBq; 10 μg) of panitumumab-DOTA-\(^{177}\)Lu to NRG mice also significantly inhibited PANC-1 tumour growth compared to control groups (TGI = 2.9 ± 0.4, P<0.001) (Fig. 4-5A). The tumour growth rate of PANC-1 xenografts in NOD/SCID administered normal saline or unlabeled panitumumab (TGI at 33 days = 6.8 ± 1.2 and 5.3 ± 0.6; respectively) in the current study was not significantly different from that previously studied in NRG mice (TGI at 33 days = 5.8 ± 0.5 and 6.1 ± 1.2; respectively) (343).
Figure 4-5. (A) Tumour growth index (TGI) in NOD/SCID mice with s.c. PANC-1 xenografts receiving three amounts (10.0 MBq; 10 µg) of panitumumab-DOTA-\textsuperscript{111}In, panitumumab-MCP-\textsuperscript{111}In, unlabeled panitumumab (10 µg) or normal saline separated by 3 weeks or in NRG mice with s.c. PANC-1 human PnCa xenografts receiving a single injection of panitumumab-DOTA-\textsuperscript{177}Lu (6.0 MBq; 10 µg). (B) Body weight index (BWI) in the same treatment and control groups. Values are mean ± SEM (n = 7-11). TGI at 43 d was significantly different (P<0.05) for \textsuperscript{111}In- and \textsuperscript{177}Lu-labeled panitumumab RICs compared to normal saline or unlabeled panitumumab controls.
4.4.6 Tumour and normal organ dosimetry

Absorbed doses in the tumour and normal organs in NOD/SCID mice with s.c. PANC-1 tumours after i.v. injection of 10 MBq (10 μg) of panitumumab-DOTA-\(^{111}\)In, panitumumab-MCP-\(^{111}\)In or in NRG mice administered with 6 MBq (10 μg) of panitumumab-DOTA-\(^{177}\)Lu are shown in Table 4-2. The normal organ doses were estimated based on the cumulative radioactivity in source organs determined in biodistribution studies (Figure 4-6) and using mouse organ S-values (323). The tumour doses were calculated using the sphere model in OLINDA/EXM (352) and the measured tumour size. There was no significant difference between the tumour dose for panitumumab-MCP-\(^{111}\)In and panitumumab-DOTA-\(^{111}\)In (2.8 ± 0.7 Gy vs 6.0 ± 2.2 Gy, respectively; P>0.05). However, for a single injection of \(^{177}\)Lu-labeled panitumumab-DOTA the absorbed doses in the tumour (11.5 ± 4.9 Gy) and normal organs were significantly higher compared to three injections of panitumumab-MCP-\(^{111}\)In or panitumumab-DOTA-\(^{111}\)In. The absorbed dose in the whole body from panitumumab-DOTA-\(^{177}\)Lu was 4.6-fold and 5.6--fold higher for panitumumab-MCP-\(^{111}\)In and panitumumab-DOTA-\(^{111}\)In, respectively. The accumulated radioactivity over time in the tumour and normal organs was lower for the \(^{111}\)In-labeled RICs compared to \(^{177}\)Lu-labeled panitumumab-DOTA (Figure 4-6) leading to lower absorbed doses in the tumour and normal organs. The highest normal organ absorbed doses from all the RICs were deposited in the kidneys and spleen followed by the pancreas and liver (Table 4-2). Due to the higher liver and spleen uptake, the absorbed doses in these organs from panitumumab-MCP-\(^{111}\)In was 2.3--fold and 1.9-fold significantly higher compared to panitumumab-DOTA-\(^{111}\)In (10.3 ± 0.6 Gy vs 4.6± 0.3 Gy; P<0.01 and 19.5 ± 3.8 Gy vs 10.3 ± 3.6 Gy; P<0.05 respectively).
Figure 4-6. Radioactivity (not corrected for decay) in selected organs at 24, 72, 120 and 168 h post i.v. injection of a single amount (6 MBq; 10 μg) of panitumumab-DOTA-\(^{177}\)Lu in NRG mice or panitumumab-DOTA-\(^{111}\)In (10 MBq ; 10 μg) or panitumumab-MCP-\(^{111}\)In (10 MBq ; 10 μg) in NOD/SCID mice. The cumulative radioactivity up to 168 h (AUC\(_{0-168h}\); Bq × sec) was obtained from the area under the curve integrated using GraphPad Prism Ver. 4.0. By assuming that there was no further biological elimination from the organ, the radioactivity at the last time point was divided by the decay constant of \(^{177}\)Lu (0.004345 h\(^{-1}\)) or \(^{111}\)In (0.0107 h\(^{-1}\)) to calculate the AUC\(_{168h-\infty}\). The total AUC\(_{0h-\infty}\) was used to estimate the radiation absorbed doses.
Table 4-3. Estimated absorbed doses in the tumour and normal organs in mice with s.c. PANC-1 xenografts injected with $^{111}$In- or $^{177}$Lu-RICs based on the $\text{AUC}_{0h-\infty}$ determined in biodistribution studies at different time points (24, 72, 120 and 168 h p.i.; Figure 4-6).

<table>
<thead>
<tr>
<th>Organ</th>
<th>$^{177}$Lu (Single injection × 6 MBq)</th>
<th>$^{111}$In (Three injections × 10 MBq)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Panitumumab-DOTA</td>
<td>Panitumumab-MCP</td>
</tr>
<tr>
<td>Heart</td>
<td>3.5 ± 0.3</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>Lungs</td>
<td>4.4 ± 1.0</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>Liver</td>
<td>6.9 ± 1.6</td>
<td>10.3±0.6</td>
</tr>
<tr>
<td>Spleen</td>
<td>10.2 ± 2.3</td>
<td>19.5±3.8</td>
</tr>
<tr>
<td>Pancreas</td>
<td>9.2 ± 5.2</td>
<td>9.6±3.0</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.0 ± 0.2</td>
<td>2.8±0.2</td>
</tr>
<tr>
<td>Intestine</td>
<td>1.9 ± 0.3</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>Kidneys</td>
<td>19.3 ± 3.1</td>
<td>8.4±1.6</td>
</tr>
<tr>
<td>Whole Body</td>
<td>2.0 ± 0.1</td>
<td>0.4±0.0</td>
</tr>
<tr>
<td>Tumour $^{b}$</td>
<td>11.5 ± 4.9</td>
<td>2.8±0.7</td>
</tr>
</tbody>
</table>

---

$^{a}$ Estimated using $D = \tilde{A} \times S$ equation and mouse S-values (323).

$^{b}$ Estimated using the sphere model in OLINDA/EXM, and the measured tumour diameter.
4.5 Discussion

We report here a promising new RIT approach for PnCa that exploits the overexpression of EGFR on almost all (>90%) tumours (308) and a clinically used fully human anti-EGFR mAb, panitumumab (Vectibix, Amgen). Panitumumab was linked to either two MCPs that present 13 DOTA chelators or to two DOTA chelators to complex the β-emitter, $^{177}$Lu or the Auger electron emitter, $^{111}$In. The MCP with multiple DOTA chelators can provide labeling at high SA (257) and may overcome limited delivery of the RICs in PnCa due to stromal barriers (353). We previously showed the therapeutic efficacy of $^{177}$Lu-labeled panitumumab-MCP on PANC-1 cells and on s.c. PANC-1 xenografts in NRG mice. In the current study we now evaluated the RIT properties of $^{111}$In-labeled panitumumab-MCP and compared these with $^{111}$In- or $^{177}$Lu-labeled panitumumab-DOTA. At all amount of radioactivity, panitumumab-DOTA-$^{177}$Lu was more potent in vitro for killing PANC-1 cells than $^{111}$In-labeled RICs (Figure 4-1) and its toxicity was not different from the previously studied panitumumab-MCP-$^{177}$Lu conjugates under the same conditions (343). This greater cytotoxicity of $^{177}$Lu vs. $^{111}$In was associated with formation of more DNA DSBs in the cell nucleus assessed by immunofluorescence for γ-H2AX (Figure 4-2), as well as higher absorbed doses in the nucleus of PANC-1 cells for $^{177}$Lu than $^{111}$In (Table 4-1 and 4-2). EGFR-specific cell binding of panitumumab-DOTA-$^{177}$Lu conjugates was confirmed by the lower cell-associated radioactivity compared to non-specific hIgG-DOTA-$^{177}$Lu or panitumumab-DOTA-$^{177}$Lu in presence of excess unlabeled panitumumab (21.9–fold or 13.7–fold lower; respectively) (Figure 4-3). The dose deposited in the nucleus of PANC-1 cells was significantly lower for non-specific hIgG-DOTA-$^{177}$Lu or panitumumab-DOTA-$^{177}$Lu co-incubated with excess panitumumab to block EGFR than panitumumab-DOTA-$^{111}$In, panitumumab-MCP-$^{111}$In or panitumumab-DOTA-$^{177}$Lu (Table 4-1). In contrast to panitumumab-MCP-$^{177}$Lu conjugates, subcellular fractionation study showed no changes with time in the proportion of cell-bound radioactivity on the membrane, in the cytoplasm or the nucleus for panitumumab-DOTA-$^{177}$Lu, suggesting more efficient internalization of panitumumab-MCP-$^{177}$Lu into PANC-1 cells than panitumumab-DOTA-$^{177}$Lu (343). However, the lower cellular binding of panitumumab-MCP-$^{177}$Lu compared to panitumumab-DOTA-$^{177}$Lu resulted in a 2–fold lower dose deposited in the nucleus of PANC-1. The limited cellular binding of panitumumab-MCP-$^{177}$Lu may be due to the steric hindrance in EGFR binding between the
molecules of panitumumab-MCP-\textsuperscript{177}Lu (50-fold molar excess) since these have larger hydrodynamic radius compared to panitumumab-DOTA-\textsuperscript{177}Lu (7.1 nm vs. 5.1 nm; respectively) (257). It should be mentioned that saturation binding assays that were performed at lower concentrations of the RICs (0.1 up to 12.5-fold molar excess) revealed similar binding affinity for radiolabeled panitumumab-MCP and panitumumab-DOTA RICs (257).

The normal tissue toxicity of panitumumab-DOTA-\textsuperscript{111}In, panitumumab-DOTA-\textsuperscript{177}Lu and panitumumab-MCP-\textsuperscript{111}In was assessed to confirm that the amounts selected for RIT studies would be safe to administer. Analysis of blood samples obtained from non-tumour bearing NOD/SCID mice 14 days after administration of panitumumab-MCP-\textsuperscript{111}In and panitumumab-DOTA-\textsuperscript{111}In (10 MBq; 10 μg) showed no hematopoietic, liver, or kidney toxicity compared to control NOD/SCID mice receiving saline as measured by CBC, ALT and Cr (Figure 4-4). Similarly, the normal tissue toxicity was assessed in non-tumour bearing NRG mice 14 days after administration of panitumumab-DOTA-\textsuperscript{177}Lu (6.0 MBq; 10 μg) or panitumumab-MCP-\textsuperscript{177}Lu (6.0 MBq; 10 μg) or saline. Hematology and serum biochemistry analysis for NRG mice administered panitumumab-MCP-\textsuperscript{177}Lu (6.0 MBq; 10 μg) and control NRG mice administered normal saline were published in our previous study (343). Here we confirmed the safety of panitumumab-DOTA-\textsuperscript{177}Lu (6.0 MBq; 10 μg) in NRG mice, which caused no significant decrease in CBC, or increase ALT and Cr (Figure 4-4 compared to previously reported values for panitumumab-MCP-\textsuperscript{177}Lu (6.0 MBq; 10 μg) or normal saline injected NRG mice (343).

Administration of a single amount (6.0 MBq; 10 μg) of panitumumab-DOTA-\textsuperscript{177}Lu or three amounts (10.0 MBq; 10 μg) of panitumumab-MCP-\textsuperscript{111}In or panitumumab-DOTA-\textsuperscript{111}In separated by 3 weeks significantly delayed tumour growth in NOD/SCID or NRG mice with s.c. PANC-1 tumours (TGI at 43 days = 2.9 ± 0.4, 3.0 ± 0.4 and 3.9 ± 0.3, respectively; P>0.05) compared to saline and unlabeled panitumumab (TGI = 9.8 ± 1.6, 9.9 ± 1.4, respectively) (Figure 4-5). Dosimetry (Table 4-3) estimated based on the biodistribution of the RICs (Figure 4-6) revealed that at three injections of \textsuperscript{111}In-RICs (total 30 MBq) or one injection of \textsuperscript{177}Lu-RICs (6 MBq), the absorbed dose in the tumour for panitumumab-DOTA-\textsuperscript{177}Lu was 11.5 ± 4.9 Gy but these RICs caused the same growth inhibitory effect on PANC-1 tumours as 6.0 ± 2.2 Gy deposited by panitumumab-DOTA-\textsuperscript{111}In and 2.8 ± 0.7 Gy deposited by panitumumab-MCP-\textsuperscript{111}In. We previously studied the tumour growth inhibition caused by a single administration of (6 MBq; 10
μg) panitumumab-MCP-\(^{177}\)Lu compared to normal saline or unlabeled panitumumab in NRG mice engrafted s.c. with PANC-1 xenografts and the results were reported as the mean TGI ± SEM at 33 days (343). The mean TGI at 33 days calculated for panitumumab-DOTA-\(^{177}\)Lu in this study is similar to that previously reported for panitumumab-MCP-\(^{177}\)Lu (TGI = 1.8 ± 0.3 vs. 2.5 ± 0.3, respectively; P> 0.05) which can be explained by the similar absorbed doses in the tumour (11.5 ± 4.9 Gy vs. 12.3 ± 0.9 Gy; P>0.05). Panitumumab-DOTA-\(^{177}\)Lu and panitumumab-MCP-\(^{177}\)Lu significantly delayed tumour growth in NRG mice compared to normal saline and unlabeled panitumumab (TGI = 5.8 ± 0.5 and TGI = 6.6 ± 1.1; P<0.05). Panitumumab neither decreased the CS of PANC-1 cells in vitro (Figure 4-1) nor delayed tumour growth of PANC-1 xenografts in vivo (Figure 4-5), likely due to a downstream mutation in KRAS (329) that results in constitutive EGFR activation.

The equivalent tumour growth-inhibitory effects of \(^{111}\)In and \(^{177}\)Lu in vivo despite 2-4 fold higher absorbed doses in PANC-1 tumours deposited by panitumumab-DOTA-\(^{177}\)Lu than panitumumab-DOTA-\(^{111}\)In or panitumumab-MCP-\(^{111}\)In; respectively (Table 4-3), could be explained by tumour hypoxia. The DNA damaging effects of β-particles rely on reactive oxygen species (ROS) while AEs are a form of high LET radiation that is cytotoxic under both normoxic and hypoxic conditions (191). PANC-1 tumours in SCID mice have been reported to be hypoxic which caused resistance to γ-irradiation (354) and may similarly cause resistance to β-particles. In contrast, in the well-oxygenated cell culture system used in vitro, there is no hypoxia that would restrict the effectiveness of the β-particles emitted by \(^{177}\)Lu. In addition, the cross-fire effect from the 2 mm range β-particles contributes to absorbed dose in PANC-1 cells from panitumumab-DOTA-\(^{177}\)Lu in the medium in vitro, whereas there is a more limited cross-fire effect from \(^{111}\)In, mainly from the low ionizing but penetrating γ-photon emissions (Table 4-1). AEs have a higher relative biological effectiveness (RBE) than β-particles due to their high LET, which means that at the same radiation dose, AEs are more damaging (191). Moreover, the tumour absorbed doses were estimated from the cumulative radioactivity in the tumour (A̅s) and OLINDA/EXM macrodosimetry (352). Thus, these macrodoses do not reflect the subcellular doses deposited by \(^{111}\)In, especially in the cell nucleus which would be more important for predicting the effectiveness of panitumumab-DOTA-\(^{111}\)In.

It is challenging to compare β-particle or AE-emitters for RIT and it has been suggested that
these may be best compared using administered amounts of radioactivity that cause equivalent normal tissue toxicity (355). In our study we were most interested to compare $^{177}$Lu- and $^{111}$In-labeled RICs at administered amounts that caused no normal tissue toxicity. Nonetheless, it should be noted that similar antitumour effects were obtained after administration of three non-toxic amounts of $^{111}$In-labeled panitumumab-DOTA and panitumumab-MCP (total = 30 MBq; 30 µg) but only one administration of $^{177}$Lu-labeled panitumumab-DOTA (6 MBq; 10 µg) (Figure 4-5). In a previous study, $^{177}$Lu-labeled RICs bispecific for HER2 and EGFR were more effective than $^{111}$In-labeled RICs, but both inhibited the growth of human breast cancer xenografts co-expressing these receptors in mice (356).

Targeting overexpression of EGFR for RIT of PnCa may cause some normal tissue toxicity since EGFR are displayed at low levels on most epithelial tissues and moderately by the liver and kidneys (51, 336). Importantly, <3% of hematopoietic stem cells are EGFR-positive (357) which should minimize hematopoietic toxicity, particularly for $^{111}$In-labeled RICs that require binding and internalization for the AEs to be damaging to DNA (10). The 2 mm range β-particles emitted by $^{177}$Lu may also cause non-specific bone marrow toxicity from a cross-fire effect, but our results revealed no hematopoietic, liver or kidney toxicity in mice administered $^{111}$In- or $^{177}$Lu-labeled RICs at the amounts studied (Figure 4-4). Nonetheless, it should be noted that panitumumab does not bind to mouse EGFR (358), and EGFR-specific normal tissue toxicities would need to be evaluated in a Phase 1 clinical trial. Previously, we found that $^{111}$In-EGF caused no hematopoietic, liver or kidney toxicity at administered amounts up to 44.4 MBq in mice or 85.1 MBq in rabbits (359) and up to 2,290 MBq in humans in a Phase I trial (360). Our results suggest that panitumumab-DOTA-$^{111}$In and panitumumab-MCP-$^{111}$In as well as panitumumab-DOTA-$^{177}$Lu are promising agents for RIT of EGFR positive cancers such as PnCa. However the MCP conjugates might have limitations over the DOTA conjugates due to their higher liver and spleen uptake, therefore DOTA conjugates might be more promising for RIT with $^{111}$In. There was no significant difference in the size of tumours treated with $^{111}$In and $^{177}$Lu but nanometer range AEs emitted by $^{111}$In might be more potent for treatment of small volume disease, while $^{177}$Lu could be used for larger tumours (361). Although all the aforementioned RICs provided strong tumour growth inhibition in mice without normal tissue toxicity, the whole body dose, which is a predictor of bone marrow toxicity was much higher for $^{177}$Lu than $^{111}$In. This would limit the ability to further escalate the administered amount of $^{177}$Lu,
but the amount of $^{111}$In could be further increased without exceeding a whole body dose of 2 Gy (limit).

4.6 Conclusion

We found some differences in the relative potency of $^{111}$In vs. $^{177}$Lu comparing in vitro and in vivo studies that may be due to hypoxia. On a per dose basis $^{111}$In was much more effective than $^{177}$Lu in vivo. In addition, $^{111}$In deposits lower doses in normal organs than $^{177}$Lu and this may allow further escalation of the administered amount of $^{111}$In, while the whole body dose for $^{177}$Lu doesn’t allow further escalation in the administered amount because of potential bone marrow toxicity. Also, the higher liver and spleen uptake of the MCP conjugates makes these less attractive for RIT with $^{111}$In than the DOTA conjugates.

4.7 Acknowledgments

Supported by grants from the Canadian Cancer Society Research Institute with funds from the Canadian Cancer Society and from the Cancer Research Society. S. Aghevlian received scholarships from the Strategic Training in Transdisciplinary Radiation Science for the 21st Century (STARS21) strategic training program at the University Health Network supported by the Terry Fox Foundation and from the Centre for Pharmaceutical Oncology (CPO) at the University of Toronto.
Chapter 5: Summary and Future Directions
Thesis Conclusion and Summary of Findings

The overall aim of this thesis research was to develop and characterize novel theranostics for imaging and systemic RIT of PnCa using anti EGFR mAb panitumumab labeled with the Auger electron emitter, $^{111}$In or $\beta$-particle emitter, $^{177}$Lu. Panitumumab was modified with either DOTA chelators or with HyNIC-MCP that harbours 13 pendant DOTA chelators and 10 PEG moieties to reduce liver and spleen uptake. The availability of multiple chelators on these polymers to complex radiometals allow for dual labeling with $^{111}$In and $^{177}$Lu, as well as more reproducible labeling efficiency and also achieving a higher SA which could provide higher toxicity towards cancer cells.

The overall conclusions of the research described in this thesis are:

I) $^{111}$In- or $^{177}$Lu-labeled panitumumab-DOTA and panitumumab-MCP RICs were constructed at high labeling yield and at high SA while preserving immunoreactivity toward EGFR. MCP increases the SA of $^{111}$In and $^{177}$Lu dual labeled panitumumab up to 12.5-fold compared to DOTA conjugation. EGFR-mediated tumour uptake of panitumumab-DOTA and panitumumab-MCP as well as the EPR effect associated with the larger hydrodynamic radius of MCP conjugates than DOTA conjugates (7.3 nm vs 5.5 nm; respectively) allowed visualization of EGFR overexpressing PANC-1 tumours in mice using microSPECT/CT imaging.

II) Panitumumab-MCP-$^{177}$Lu reduced the CS of PANC-1 cells in vitro by 7.7-fold at 1.2 MBq (2.5 nmol/L) compared to unlabeled panitumumab due to a high absorbed dose in the nucleus (3.84 Gy) causing 3.8-fold higher $\gamma$-H2AX foci in PANC-1 cells. Intravenous (i.v.) injection of 6 MBq of panitumumab-MCP-$^{177}$Lu (10 μg) delivered a dose of 12.5 Gy to PANC-1 tumours and significantly inhibited tumour growth in NRG mice compared to the control groups including saline, unlabeled panitumumab and hIgG-MCP-$^{177}$Lu (TGI at 33 days $= 2.3 \pm 0.2$ vs. $5.8 \pm 0.5$, $6.0 \pm 1.6$ and $4.0 \pm 0.7$ respectively) without any observable normal tissue toxicity.

III) Panitumumab-DOTA-$^{177}$Lu reduced the CS of PANC-1 cells in vitro by 6.4-fold and 5-fold at 1.2 MB (2.5 nmol/L) compared to panitumumab-DOTA-$^{111}$In and panitumumab-MCP-$^{111}$In due to a higher absorbed dose in the nucleus (8.06 Gy v.s 2.2 Gy and 1.17 Gy; respectively) causing 2.7-fold higher $\gamma$-H2AX foci in PANC-1 cells.
Intravenous (i.v.) injection of 6 MBq of panitumumab-DOTA-\(^{177}\)Lu (10 µg) delivered 2-4 fold higher absorbed dose to PANC-1 tumours compared to 3 amounts of 10 MBq of \(^{111}\)In-labeled RICs (10 µg), however as measured by TGI at 43 days p.i. of panitumumab-DOTA-\(^{177}\)Lu, panitumumab-DOTA-\(^{111}\)In and panitumumab-MCP-\(^{111}\)In tumour growth was similarly delayed in mice with s.c. PANC-1 xenografts compared to control mice treated with saline and unlabeled panitumumab (2.9 ± 0.43, 3.03 ± 0.45 and 3.87 ± 0.35; respectively vs. 9.83 ± 1.56 and 9.87 ± 1.38; respectively) without any observable normal tissue toxicity.

5.1.1 Chapter 2

This chapter characterized the in vitro and in vivo properties of \(^{111}\)In- or \(^{177}\)Lu- labeled panitumumab-DOTA and panitumumab-MCP conjugates and their ability to visualize PANC-1 tumours in NOD/scid mice implanted s.c. with PANC-1 xenografts using microSPECT/CT imaging. Panitumumab was modified with S-4FB through lysine groups in order to install aldehyde groups for derivatization with HyNIC-MCP (two MCPs/panitumumab). For comparison, using similar linkage chemistry, DOTA-NHS ester (succinimidyl ester) was selected for reaction with lysines on panitumumab for synthesis of panitumumab-DOTA conjugates (2 DOTA/panitumumab). When 1 µg the RICs were dual-labeled with \(^{111}\)InCl\(_3\) (7.8 MBq) and \(^{177}\)LuCl\(_3\) (5.7 MBq), the SA of panitumumab-MCP conjugates was 12.7-fold and 9.3-fold higher compared to panitumumab-DOTA conjugates. Panitumumab-MCP-\(^{177}\)Lu and panitumumab-DOTA-\(^{177}\)Lu exhibited saturable binding to EGFR overexpressing MDA-MB-468 cells (1.3 × 10\(^6\) receptors/cell) that was displaced by an excess of unlabeled panitumumab, demonstrating specific EGFR-mediated binding. There were no significant differences in the EGFR binding affinity of Panitumumab-MCP-\(^{177}\)Lu and panitumumab-DOTA-\(^{177}\)Lu (Kd = 2.2 ± 0.6 nmol/L and 1.0 ± 0.4 nmol/L, respectively). \(^{111}\)In- or \(^{177}\)Lu-labeled panitumumab-MCP or panitumumab-DOTA were stable to loss of radiometal when challenged in vitro with a 500-fold excess of EDTA or when incubated in human plasma at 37\(^\circ\)C for up to 1 week. Comparison of the elimination of radioactivity from the body in NOD/SCID mice engrafted s.c PANC-1 xenografts and excretion into the urine and feces up to 72 h p.i. of panitumumab-MCP-\(^{111}\)In or panitumumab-DOTA-\(^{111}\)In showed both RICs were slowly eliminated from the body with 40-45% of the injected dose eliminated over 72h. Most of the radioactivity eliminated from the body
thorough the urinary system. These results suggest that there was no instability of the RICs introduced by use of the MCP for labeling with $^{111}$In compared to DOTA. Biodistribution study in NOD/SCID mice engrafted s.c PANC-1 xenografts at 72h p.i. showed no significant difference in the tumour uptake of panitumumab-MCP-1$^{111}$In and panitumumab-DOTA-1$^{111}$In (6.9 ± 1.3 vs 6.6 ± 3.3%ID/g, respectively). No significant differences were observed in normal tissue localization between the two RICs, except for the liver which was 3-fold higher for panitumumab-MCP-111In. EGFR-mediated tumour uptake of panitumumab-DOTA-1$^{111}$In was confirmed by 3.5-fold reduced uptake of IgG-DOTA-1$^{111}$In, however similar tumour uptake of nonspecific hIgG-MCP (5.4 ± 0.3%ID/g) to panitumumab-MCP-1$^{111}$In suggests EPR-mediated tumour uptake due to the larger hydrodynamic radius of panitumumab-MCP than panitumumab-DOTA (7.3 nm vs 5.5 nm; respectively). PANC-1 tumours were imaged by microSPECT/CT at 72 h p.i. of panitumumab-DOTA-1$^{111}$In, panitumumab-MCP-1$^{111}$In or hIgG-MCP-1$^{111}$In. It was concluded that 1$^{111}$In- or 177Lu- labeled DOTA or MCP conjugated to panitumumab are stable RICs with similar biodistribution profiles and could be useful theranostic agents for EGFR-positive PnCa.

5.1.2 Chapter 3

This chapter evaluated the safety and efficacy of systemic RIT of s.c. PANC-1 human PnCa xenografts in NRG mice using anti-EGFR panitumumab linked to MCP that presents 13 DOTA chelators to complex the β-emitter, 177Lu. Confocal immunofluorescence microscopy for γ-H2AX showed that panitumumab-MCP-177Lu caused more DNA DSBs in the nucleus of PANC-1 cells as the SA increased (0.3, 0.6, 1.2 MBq; SA = 46.7, 93.5, 187.0 MBq/nmole). Despite the ineffectiveness of unlabeled panitumumab for killing PANC-1 cells, panitumumab-MCP-177Lu reduced the CS of PANC-1 cells by 7.7-fold at 1.2 MBq, partly due to deposition of 3.84 Gy in the nucleus of PANC-1 cells as measured by subcellular distribution of panitumumab-MCP-177Lu at different time points from 1 to 24 h following incubation. Prior to RIT, toxicity study showed that administration of panitumumab-MCP-177Lu (6 MBq; 10 μg) to NRG mice caused no significant change in body weight, CBC or ALT and Cr compared to NRG mice treated with normal saline. RIT was performed in NRG mice with s.c. PANC-1 tumours injected i.v. with 6 MBq (10 μg) of panitumumab-MCP-177Lu, hIgG-MCP-177Lu (6 MBq; 10 μg), unlabeled panitumumab (10 μg) or normal saline (100 μL). The tumour growth index (TGI) was compared.
Panitumumab-MCP-$^{177}$Lu strongly inhibited tumour growth in NRG mice (TGI = 2.3 ± 0.2) compared to normal saline treated mice (TGI = 5.8 ± 0.5; P<0.01). As expected due to KRAS mutation, unlabeled panitumumab had no effect on tumour growth (TGI = 6.0 ± 1.6; P>0.05). IgG-MCP-$^{177}$Lu delayed tumour growth but it wasn’t as effective as Panitumumab-MCP-$^{177}$Lu (TGI = 4.0 ± 0.7; P>0.05). Tumour and normal organ doses were estimated based on biodistribution studies at 1, 3, 5 and 7 days p.i. The absorbed dose of PANC-1 tumours was 12.3 Gy. The highest normal organ doses were absorbed by the pancreas, liver, spleen, and kidneys. Due to presence of multiple DOTA chelators on the MCP, panitumumab-MCP conjugates can be labeled at very high SA, however injecting a large radioactivity will exceed the maximum tolerated injected dose in mice. Therefore, in this study, the injected amount limit was 6 MBq. We conclude that EGFR-targeted RIT with panitumumab-MCP-$^{177}$Lu was able to overcome resistance to panitumumab in KRAS mutant PANC-1 tumours in NRG mice and may be a promising approach to treatment of PnCa in humans.

5.1.3 Chapter 4

Chapter 4 focused on comparing the therapeutic efficacy of the β-particle emitter, $^{177}$Lu and Auger electron-emitter, $^{111}$In targeted to EGFR positive PANC-1 cells in vitro or to s.c. PANC-1 xenografts in mice by exploiting DOTA-conjugated or MCP-conjugated panitumumab. The clonogenic survival of PANC-1 cells was determined following exposure to increasing radioactivity of (0.3-1.2 MBq; 2.5 nmole/L) panitumumab-DOTA-$^{177}$Lu, panitumumab-DOTA-$^{111}$In or panitumumab-DOTA-$^{111}$In (2.5 nmole/L) for 16 h then culturing for 12 days. At all amounts of radioactivity panitumumab-DOTA-$^{177}$Lu was more cytotoxic than $^{111}$In-labeled RICs. In a separate study, PANC-1 cells were incubated with the RICs under the same condition and only for 16 h to assess the intensity of γ-H2AX representing unrepaired DNA DSBs by immunofluorescence. Panitumumab-DOTA-$^{177}$Lu induced more DNA DSBs in the nucleus of PANC-1 cells compared to $^{111}$In-labeled RICs. At 1.2 MBq, panitumumab-DOTA-$^{177}$Lu induced 2.7-fold and 1.56-fold higher γ-H2AX foci than panitumumab-DOTA-$^{111}$In and panitumumab-MCP-$^{111}$In; respectively, which is attributed to the higher absorbed dose in the nucleus by panitumumab-DOTA-$^{177}$Lu (8.06 Gy) compared to panitumumab-DOTA-$^{111}$In and panitumumab-MCP-$^{111}$In (2.28 Gy and 1.17 Gy; respectively) as measured by subcellular distribution of conjugates in PANC-1 cells. In the previous chapter we showed that
panitumumab-MCP-\(^{177}\)Lu deposited 3.84 Gy in the nucleus of PANC-1 cells. It’s not clear why the cellular binding of panitumumab-MCP is lower than panitumumab-DOTA however it’s assumed that the larger hydrodynamic size of panitumumab-MCP-\(^{177}\)Lu compared to panitumumab-DOTA-\(^{177}\)Lu might play a role. Stronger steric hindrance between the molecules of panitumumab-MCP-\(^{177}\)Lu (50-fold molar excess) might lower the cellular binding of panitumumab-MCP-\(^{177}\)Lu to PANC-1 cells resulting in a lower absorbed dose in the nucleus.

Administration to NRG mice of panitumumab-DOTA-\(^{177}\)Lu (6 MBq; 10 \(\mu\)g) or to NOD/SCID mice of panitumumab-DOTA-\(^{111}\)In or panitumumab-MCP-\(^{111}\)In (10 MBq; 10 \(\mu\)g) caused no significant change in body weight, decreased CBC or increases in ALT or Cr compared to normal saline treated NOD/SCID or NRG mice after 14 days. For RIT, NOD/SCID mice with s.c. PANC-1 tumours received three amounts (10 MBq; 10 \(\mu\)g) of \(^{111}\)In-labeled RICs separated by 3 weeks, whereas tumour bearing NRG mice received a single amount (6 MBq; 10 \(\mu\)g) of panitumumab-DOTA-\(^{177}\)Lu. Control NOD/SCID mice were treated with normal saline or unlabeled panitumumab (10 \(\mu\)g). TGI at 43 day was calculated to include more than 3 surviving mice in each group. This value for panitumumab-DOTA-\(^{111}\)In or panitumumab-MCP-\(^{111}\)In was not significantly different from that for panitumumab-DOTA-\(^{177}\)Lu (TGI = 3.02 ± 0.45, 3.87 ± 0.35 and 2.9 ± 0.43; respectively, P>0.5), whereas TGI at 43 days for both RICs were significantly lower than that for normal saline and unlabeled panitumumab (TGI = 9.83 ± 1.56; and TGI = 9.87 ± 1.38; respectively P<0.001). Tumour and normal organ doses were also estimated based on biodistribution studies in NRG mice and NOD/SCID mice at different time points. Absorbed doses in PANC-1 tumours were 11.52 ± 4.93 for panitumumab-DOTA-\(^{177}\)Lu and 2.79 ± 0.75 and 6.03 ± 2.22 for Panitumumab-MCP-\(^{111}\)In and panitumumab-DOTA-\(^{111}\)In; respectively. The highest normal organ doses were received by the kidneys, spleen and pancreas. Although studied under the same conditions, \(^{111}\)In deposited lower doses in the tumour and normal organs, but the \(^{111}\)In-labeled RICs were still more effective than the \(^{177}\)Lu-RICs, possibly because Auger electrons have higher RBE, thus at lower radiation doses they can be as effective or more effective than \(\beta\)-particles (355).

In this study the mean TGI at 33 days for panitumumab-DOTA-\(^{177}\)Lu was not significantly different from that for panitumumab-MCP-\(^{177}\)Lu (TGI = 1.83 ± 0.25 vs. TGI = 2.5 ± 0.3; P>0.05) which can be explained by their equal absorbed dose in the tumour (11.52 ± 4.93 Gy vs. 12.33 ± 0.86 Gy; P>0.05). Panitumumab-DOTA-\(^{177}\)Lu and panitumumab-MCP-\(^{177}\)Lu
significantly delayed tumour growth in NRG mice compared to saline and unlabeled panitumumab control mice (TGI = 5.8 ± 0.5 and TGI = 6.6 ± 1.1; P<0.05). In chapter 4 it was shown that panitumumab-DOTA or panitumumab-MCP conjugates labeled with $^{177}$Lu or $^{111}$In are promising RIT agents for treatment of PnCa in humans.

5.1.4 Future directions

In the past decade the rate of PnCa diagnosis has been increasing steadily and despite research, there remain high mortality rates from this disease (362). The safety and efficacy identified in animal studies is not generally translated to human trials because many of the animal models fail to faithfully recapitulate the dynamic of human cancer. Studies have reported that compared with subcutaneous xenograft models, orthotopic xenograft models in which tumour xenografts are implanted into the natural organ, better simulate clinical cancer in terms of metastasis and invasion, which may be attributed to the difference in microenvironment and lymphatic vessel densities (363, 364). In this project the immunohistochemical studies showed that the subcutaneous PANC-1 xenografts have a simple structure with homogeneous expression of EGFR throughout the tumour (Appendix B). As opposed to subcutaneous xenograft models, the tumour microenvironments of orthotopic xenograft models are more comparable to those in humans and therefore rendering these models more relevant in predicting clinical outcomes in humans (362). Furthermore, frequent metastases are observed in orthotopic xenograft models. Studies have shown that up to 60% of orthotopic models of PnCa can disseminate to other organ sites (365). Derosier et al. suggested in PnCa research the necessary second step is to employ orthotopic xenograft mouse models to further validate results established in subcutaneous xenograft mouse models (366). In the future, orthotopic tumour xenograft mouse models for PnCa is recommended to further study the SPECT theranostics.

5.1.4.1 Bioluminescence orthotopic model of PANC-1

In PnCa, tumour cells with the ability to enter the blood circulation, i.e. circulating tumour cells (CTCs) are responsible for development of metastases in distant organs particularly the liver, lung and skeletal system (367). Recent studies have shown increasing interest in the application of the luciferase-luciferin reaction for metastasis detection. Our lab has recently shown that tail vein injection of luciferase-labeled MDA-MB-231 human breast cancer cells into NOD-SCID mice and CD1 nude mice leads to metastases to the brain, lungs, liver, spleen and kidneys which
are visualized by injection of luciferin prior to bioluminescence imaging. Similarly, PANC-1 human PnCa cells can be tagged with luciferase (368-370) and administered i.v. into mice to establish detectable metastases. The therapeutic efficacy of RICs particularly panitumumab-DOTA-\(^{111}\)In and panitumumab-MCP-\(^{111}\)In in controlling metastases could be evaluated by monitoring metastases using bioluminescence imaging. The high LET (4-26 keV/\(\mu\)m) of Auger electrons emitted by \(^{111}\)In which travel only a short distance (nm to \(\mu\)m) in tissues makes \(^{111}\)In suitable for eradicating micrometastases and CTCs. Since the role of imaging clinically would be to detect metastases in order to better stage patients, the SPECT imaging properties of the RICs can be studies in these metastatic as well as orthotopic models.

### 5.1.4.2 Evaluation of RICs in combination with other anti-cancer agents

According to the challenges of PnCa treatment mostly because of hypoxia (124, 371-373) and low drug delivery [hypovascularity (71, 124, 371-373), stromal barrier (36, 297, 374, 375)] combination of hypoxia-targeting drugs like evofosfamide (formerly known as TH-302) to increase the sensitivity to radiation (376, 377) might be useful to improve the outcome of RIT. RIT of PANC-1 tumour in mice with panitumumab-MCP and panitumumab-DOTA conjugates labeled with \(^{111}\)In or \(^{177}\)Lu significantly delayed tumour growth in mice compared to unlabeled panitumumab, but didn’t result in tumour growth arrest. The therapeutic efficacy of these RICs alone or in combination with drugs like nab-paclitaxel and evofosfamide could be studied and compared with FOLFIRINOX or gemcitabine as the first line and second line treatment of PnCa (378).

The SPECT theranostic RICs developed here may play an important role in future clinical practice. Their ability to combine diagnostic and therapeutic capabilities may offer personalized RIT for patients with EGFR-positive tumours. Beta-particles emitted by \(^{177}\)Lu could be useful for RIT of millimetre-sized tumours whereas nanometer range Auger electrons emitted by \(^{111}\)In could be more potent for treatment of micrometastases. Furthermore low absorbed doses in the normal organs for \(^{111}\)In allow for dose escalation and more fractions. MCP provide an opportunity for labeling panitumumab at high specific activity. While these high specific activity probes have theoretical advantages in increasing the potency of the RICs, the benefit that MCP conjugates may provide patients may need to be balanced against their higher accumulation in the liver and spleen and the risk for low mass amounts to further promote uptake in these organs.
It would be necessary to test panitumumab-MCP conjugates at high specific activity side by side with panitumumab-DOTA conjugates in a first-in human Phase I clinical trial in patients with EGFR-positive tumours.
Appendices
6.1 Appendix A: Therapeutic efficacy of the $^{111}\text{In}$- and $^{177}\text{Lu}$-labeled RICs side by side

For a better comparison, this section displays all the results for $^{111}\text{In}$-labeled RICs and $^{177}\text{Lu}$-labeled RICs side by side.
Appendix A1. Clonogenic survival

Figure S1 Clonogenic survival of PANC-1 cells exposed to increasing radioactivity of $^{177}$Lu- or $^{111}$In- labeled panitumumab-MCP (2.5 nmol/L) or panitumumab-DOTA (2.5 nmol/L) or to unlabeled immunoconjugates (2.5 nmol/L) for 16 h then cultured for 12 d. Data shown are the mean ± SEM (n = 3–4). Significant differences (P<0.05) between $^{111}$In- or $^{177}$Lu- labeled RICs are indicated by the asterisks. No significant deference was observed between panitumumab-MCP-$^{111}$In and panitumumab-DOTA-$^{111}$In or between panitumumab-MCP-$^{177}$Lu and panitumumab-DOTA-$^{177}$Lu.
6.1.2 Appendix A2: DNA damage assessment using γ-H2AX assay

Figure S2. (A) Representative confocal immunofluorescence microscopy images assessing γ-H2AX foci (green) representing sites of unrepaired DNA DSBs in PANC-1 cells exposed for 16 h to 0.3, 0.6 or 1.2 MBq of $^{177}$Lu- or $^{111}$In-labeled panitumumab-DOTA or panitumumab-MCP. Cell nuclei were counterstained with DAPI (blue). In the bottom set of panels are images of PANC-1 cells exposed to growth medium alone, or unlabeled panitumumab-DOTA or panitumumab-MCP. (B) γ-H2AX foci were quantified as the integrated density/nucleus area and are shown for untreated PANC-1 cells, cells treated with unlabeled radioimmunoconjugates (RICs) or increasing amounts of $^{177}$Lu- or $^{111}$In-labeled RICs. Values shown represent the mean ± SEM (n= 3–4). Significant differences (P<0.05) are indicated by the asterisks.
6.1.3 Appendix A3: Absorbed dose in the nucleus of PANC-1 cells in vitro for $^{111}\text{In}$- or $^{177}\text{Lu}$-RICs

Table S 1. Absorbed dose in the nucleus were estimated following incubation of cells with 1.2 MBq (2.5 nmol/L) of $^{177}\text{Lu}$-RICs in the absence or presence of 50-fold molar excess of unlabeled panitumumab to block EGFR for 24 h at 37 °C.

<table>
<thead>
<tr>
<th>Source compartment</th>
<th>S (Gy/Bq×sec)</th>
<th>Panitumumab-DOTA</th>
<th>Panitumumab-DOTA+ excess panitumumab</th>
<th>hIgG-DOTA</th>
<th>Panitumumab-MCP</th>
<th>Panitumumab-MCP + excess panitumumab</th>
<th>hIgG-MCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell surface</td>
<td>$3.55 \times 10^4$</td>
<td>4.86</td>
<td>0.3</td>
<td>0.12</td>
<td>1.42</td>
<td>0.13</td>
<td>0.19</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>$4.02 \times 10^4$</td>
<td>1.18</td>
<td>0.05</td>
<td>0.03</td>
<td>0.86</td>
<td>0.13</td>
<td>0.42</td>
</tr>
<tr>
<td>Nucleus</td>
<td>$6.93 \times 10^4$</td>
<td>0.87</td>
<td>0.05</td>
<td>0.09</td>
<td>0.46</td>
<td>0.07</td>
<td>0.32</td>
</tr>
<tr>
<td>Medium</td>
<td>$1.15 \times 10^{11}$</td>
<td>1.13</td>
<td>1.13</td>
<td>1.13</td>
<td>1.13</td>
<td>1.13</td>
<td>1.13</td>
</tr>
<tr>
<td>Total</td>
<td>$8.06$</td>
<td>1.53</td>
<td>1.36</td>
<td>3.87</td>
<td>1.46</td>
<td>2.06</td>
<td></td>
</tr>
</tbody>
</table>

Table S 2. Absorbed dose in the nucleus were estimated following incubation of cells with 1.2 MBq (2.5 nmol/L) of $^{111}\text{In}$-RICs in the absence or presence of 50-fold molar excess of unlabeled panitumumab to block EGFR for 24 h at 37 °C.

<table>
<thead>
<tr>
<th>Source compartment</th>
<th>S (Gy/Bq×sec)</th>
<th>Panitumumab-DOTA</th>
<th>Panitumumab-DOTA+ excess panitumumab</th>
<th>hIgG-DOTA</th>
<th>Panitumumab-MCP</th>
<th>Panitumumab-MCP + excess panitumumab</th>
<th>hIgG-MCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell surface</td>
<td>$7.35 \times 10^5$</td>
<td>1.01</td>
<td>0.06</td>
<td>0.02</td>
<td>0.29</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>$1.03 \times 10^4$</td>
<td>0.3</td>
<td>0.01</td>
<td>0.007</td>
<td>0.22</td>
<td>0.03</td>
<td>0.1</td>
</tr>
<tr>
<td>Nucleus</td>
<td>$5.06 \times 10^4$</td>
<td>0.64</td>
<td>0.04</td>
<td>0.06</td>
<td>0.33</td>
<td>0.05</td>
<td>0.23</td>
</tr>
<tr>
<td>Medium</td>
<td>$3.39 \times 10^{12}$</td>
<td>0.33</td>
<td>0.33</td>
<td>0.33</td>
<td>0.33</td>
<td>0.33</td>
<td>0.33</td>
</tr>
<tr>
<td>Total</td>
<td>$2.28$</td>
<td>0.44</td>
<td>0.43</td>
<td>1.17</td>
<td>0.43</td>
<td>0.69</td>
<td></td>
</tr>
</tbody>
</table>
6.1.4 Appendix A4: Toxicity study

Figure S 3. Hematology and serum biochemistry analyses of NOD/SCID mice at 14 d after i.v. injection of 10.0 MBq (10 μg) of panitumumab-MCP-\(^{111}\)In, panitumumab-DOTA-\(^{111}\)In or normal saline and NRG mice administered 6.0 MBq (10 μg) of panitumumab-MCP-\(^{177}\)Lu, panitumumab-DOTA-\(^{177}\)Lu or normal saline. (A) white blood cells [WBC], (B) Red blood cells [RBC], (C) hemoglobin [Hb], (D) hematocrit [HCT], (E) platelets [PLT], (F) serum creatinine [Cr], (G) serum alanine aminotransferase [ALT], Individual values are shown as well as the mean (horizontal bars). No significant difference was observed between control and treatment groups.
6.1.5 Appendix A5: RIT study with $^{177}$Lu-RICs

Figure S4. Body weight index (BWI) in (A) NRG mice with s.c. PANC-1 human pancreatic cancer (PnCa) xenografts receiving single amount (6.0 MBq; 10 μg) of panitumumab-MCP-$^{177}$Lu, panitumumab-DOTA-$^{177}$Lu or non-specific hIgG-MCP-$^{177}$Lu or in mice receiving unlabeled panitumumab (10 μg) or normal saline. (B) Tumour growth index (TGI) in the aforementioned groups. Values are mean ± SEM. Significant differences (P<0.05) between TGI at 33 days after starting treatment between treatment groups are indicated by the asterisks. No significant difference was observed in the TGI between panitumumab-MCP-$^{177}$Lu and panitumumab-DOTA-$^{177}$Lu (2.3 ± 0.2 vs. 1.8 ± 0.25).
6.1.6 Appendix A6: RIT study with $^{111}$In-RICs

Figure S 5. Body weight index (BWI) in (A) NOD/SCID mice with s.c. PANC-1 human pancreatic cancer (PnCa) xenografts receiving radioimmunotherapy (RIT) with three amounts (10.0 MBq; 10 µg) of panitumumab-MCP-$^{111}$In, panitumumab-DOTA-$^{111}$In or non-specific hIgG-MCP-$^{111}$In separated by 3 weeks or in mice receiving unlabeled panitumumab (10 µg) or normal saline. The mice injected with started losing weight significantly since day 10 p.i. suggesting toxicity of hIgG-MCP-$^{111}$In (B). Tumour growth index (TGI) in the aforementioned groups. Values are mean ± SEM. Significant differences (P<0.05) between TGI at 43 days after starting treatment between treatment groups are indicated by the asterisks. No significant difference was observed in the TGI between panitumumab-MCP-$^{111}$In, panitumumab-DOTA-$^{111}$In (3.03 ± 0.45 vs. 3.87 ± 0.35).
6.1.7 Appendix A7: Tumour and normal organs dosimetry

Table S 3. Estimated absorbed doses in the tumour and normal organs in mice with s.c. PANC-1 xenografts injected with $^{111}$In- or $^{177}$Lu-RICs based on biodistribution study at different time points (1, 3, 5 and 7 days p.i.). Dose to infinity was calculated by dividing the radioactivity at 168 h p.i. of RICs for source organs by the decay constant for $^{177}$Lu (0.004345 h$^{-1}$) or $^{111}$In (0.0107 h$^{-1}$), assuming further elimination only by radioactive decay.

<table>
<thead>
<tr>
<th>Organ</th>
<th>$^{177}$Lu (Single injection × 6 MBq)</th>
<th>$^{111}$In (Three injections × 10 MBq)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Panitumumab-MCP</td>
<td>Panitumumab-DOTA</td>
</tr>
<tr>
<td>Heart</td>
<td>3.4 ± 0.4</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>Lungs</td>
<td>4.3 ± 0.5</td>
<td>4.4 ± 1.0</td>
</tr>
<tr>
<td>Liver</td>
<td>7.5 ± 1.5</td>
<td>6.9 ± 1.6</td>
</tr>
<tr>
<td>Spleen</td>
<td>14.8 ± 2.8</td>
<td>10.2 ± 2.3</td>
</tr>
<tr>
<td>Pancreas</td>
<td>19.3 ± 6.5</td>
<td>9.2 ± 5.2</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.9 ± 0.4</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Intestine</td>
<td>3.2 ± 1.6</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>Kidneys</td>
<td>15.7 ± 3.7</td>
<td>19.3 ± 3.1</td>
</tr>
<tr>
<td>Whole Body</td>
<td>1.40 ± 0.7</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Tumour</td>
<td>12.3 ± 0.9</td>
<td>11.52 ± 4.9</td>
</tr>
</tbody>
</table>

*The absorbed dose in the liver for panitumumab-MCP-$^{111}$In is higher than that for panitumumab-DOTA-$^{111}$In due to the higher liver uptake of panitumumab-MCP-$^{111}$In.

†No significant difference in the absorbed dose in tumour between $^{111}$In labeled panitumumab-DOTA and panitumumab-MCP.
6.2 Appendix B. Immunohistochemical staining of PANC-1 xenografts obtained from NOD/SCID mice and NRG mice for characterization of the tumour microenvironment

Methods: To compare the tumour microenvironment of human PANC-1 xenografts (n=5) developed in NRG mice with that developed in NOD/SCID mice, the density of PANC-1 cells (EGFR\(^+\)), vascular endothelial cells (CD31\(^+\)) and possible presence of fibroblasts (alpha-smooth muscle actin (\(\alpha\)-SMA\(^+\))) was determined by immunohistochemical staining (IHC). The tissues were removed and snap-frozen in FSC 22 clear frozen section media (Leica Biosystems, Germany) cooled in liquid nitrogen. 5 \(\mu\)m frozen sections were obtained and air dried at room temperature. Sections were then fixed with acetone for 10 minutes. Endogenous peroxidase and biotin activities were blocked respectively using 0.3% hydrogen peroxide (Vector Labs). The Abs, dilutions and detection kits used in this study are shown in Table S 3. After following kit instructions, color development was performed with freshly prepared DAB (DAKO). Finally, nuclei were counterstained lightly with Mayer’s Hematoxylin and coverslipped with Permount mounting medium (Fisher). Reaction products and non-reactive nuclei were visualized in brown (DAB) and blue respectively.

Table S 4. Staining of various antigens in PANC-1 tumour sections.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Clone</th>
<th>Dilution (Incubation)</th>
<th>Detection kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD 31 rat monoclonal</td>
<td>BD Pharmingen</td>
<td>MEC13.3</td>
<td>1/500 1hr</td>
<td>Anti-Rat IgG</td>
</tr>
<tr>
<td>SMA rabbit polyclonal</td>
<td>Abcam</td>
<td>N/A</td>
<td>1/4000 1hr</td>
<td>Anti-Rabbit IgG</td>
</tr>
</tbody>
</table>
Results: Similar findings were observed in NOD/SCID mice and NRG mice. Both mouse strains developed hypovascular tumours with low population of α-SMA$^+$ cells. Although α-SMA is widely used as a cancer associated fibroblast (CAF) marker, pericytes wrapping around the endothelial cells also express this marker(379) as it can be seen in Figure S 6.

Figure S 6. The expression of EGFR, CD31 and α-SMA on PANC-1 xenografts obtained from NOD/SCID mice (on the left-hand side) and NRG mice (on the right-hand side). The brown color indicates the reaction site where antibody detects the marker. No significant deference was observed in the tumour microenvironment between the two mouse strains. Homogenous EGFR expression and hypovascularity was shown by EGFR and CD31 Staining. It seems that many α-SMA positive cells are perivascular cells.
References


64. Matthew S. Irinotecan Liposome Injection Plus Fluorouracil/Leucovorin to Treat Patients With Metastatic Pancreatic Cancer: The ASCOPost; 2015 [Available from:


133. Rudin M. Molecular Imaging : Basic Principles and Applications in Biomedical Research. 2nd ed: Imperial College Press; 2005.


145. D VHD. Actionable targets in pancreatic cancer detected by immunohistochemistry (IHC), microarray (MA) fluorescent in situ hybridization (FISH), and mutational analysis. ASCO: J Clin Oncol; 2012.


300. Ng SP, Herman JM. Stereotactic Radiotherapy and Particle Therapy for Pancreatic Cancer. Cancers (Basel). 2018;10(3).


