PET Theranostics for Molecular Imaging and Radioimmunotherapy of Pancreatic Cancer

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Pharmaceutical Sciences
University of Toronto

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Abstract

Epidermal growth factor receptor (EGFR) is overexpressed in up to 90% of pancreatic cancer tumours. Panitumumab is an antibody that targets EGFR and can be radiolabeled for molecular imaging and radioimmunotherapy (RIT). This thesis aimed to develop a “theranostic” agent that could be used for both imaging and treatment of pancreatic tumours. The first study demonstrated the ability of $^{64}$Cu-panitumumab F(ab')$_2$ fragments to visualize patient-derived orthotopic pancreatic cancer xenograft mouse models with PET/CT imaging and showed the superiority of panitumumab F(ab')$_2$ over IgG or Fab fragments. Next, studies were performed involving metal-chelating polymers (MCps) to allow antibodies to carry up to 20-fold more chelation sites compared to antibodies directly derivatized with chelators for $^{64}$Cu-radiolabeling, thereby amplifying radiation delivered per antibody. This work demonstrated that the presence of pendant PEG chains on MCps resulted in longer blood circulation times compared to MCps without pendant PEG chains. The next study demonstrated that $^{64}$Cu-panitumumab F(ab')$_2$ was useful in RIT when combined with radiosensitizing agents in pancreatic cancer xenograft mouse models. Tumour regression was not achieved so the final investigation compared $^{64}$Cu and $^{177}$Lu-panitumumab F(ab')$_2$, which have similar β-emission energies but $^{177}$Lu has double the abundance of β-emissions compared to $^{64}$Cu. $^{64}$Cu-RIT and $^{177}$Lu-RIT were compared to external γ-irradiation to determine their relative biological effectiveness (RBE) in four pancreatic cancer cell lines, with different EGFR overexpression and proliferation rates. This work demonstrated that the degree of EGFR overexpression did not predict responsiveness of cells to RIT with $^{64}$Cu- or $^{177}$Lu-RIT. Also, fast growing cells responded very well to $^{64}$Cu-RIT while slow growing cells responded extremely poorly, while proliferation rate did not correlate with response to $^{177}$Lu-
RIT. In conclusion, $^{64}$Cu-panitumumab F(ab$'$)$_2$ fragments can be useful in PET imaging of pancreatic cancer tumours and that $^{177}$Lu-panitumumab F(ab$'$)$_2$ fragments are more appropriate for RIT.
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Chapter 1
Introduction

The excerpt on α-particles was written by Amanda Jean Boyle.

Figure 1.1 of this chapter has been published in Clinical Gastroenterology and Hepatology: Wong et al. Staging of pancreatic adenocarcinoma by imaging studies. 2008;6:1301-8. A link to the published paper can be found at https://www.sciencedirect.com/science/article/pii/S1542356508009919?via%3Dihub

Figure 1.10 of this chapter has been published in Annals of Surgical Oncology: Farma et al. PET/CT fusion scan enhances CT staging in patients with pancreatic neoplasms. 2008;15:2465-71. A link to the published paper can be found at https://link.springer.com/article/10.1245%2Fs10434-008-9992-0
## Introduction

### 1.1 Incidence, diagnosis, and management of pancreatic cancer

#### 1.1.1 Epidemiology and etiology of pancreatic cancer

Pancreatic cancer is among the most lethal cancers and holds the highest mortality rate among all major cancers. It is the 4\textsuperscript{th} leading cause of cancer-related death in Canada with a 5-year survival rate of 6\%, and a 75\% mortality rate within the first year after diagnosis [1]. It is estimated that pancreatic cancer is responsible for 220,000 deaths annually around the world [2]. The most common form of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC) which accounts for 80\% of pancreatic cancers [3]. Pancreatic neuroendocrine tumours (PNETS) account for 1.3–2.8\% of new pancreatic neoplasms by incidence [4]. The incidence of pancreatic cancer has been shown to be higher in developed countries, a finding that suggests that lifestyle and environment contribute to the development of pancreatic cancer [5]. Recognized risk factors that contribute to the development of pancreatic cancer include age, sex, smoking, alcohol consumption, diet, and obesity [2, 5]. Over 90\% of pancreatic cancer patients are diagnosed over the age of 50 years old. Males have been found to have a slightly higher incidence of pancreatic cancer than women. Smoking is the leading contributor to the development of pancreatic cancer and has been determined as being responsible for an estimated 25\% of pancreatic cancer cases [5]. The contribution of alcohol consumption to the development of pancreatic cancer lies in that alcohol may initiate an inflammatory response that causes chronic pancreatitis or diabetes mellitus [6, 7]. In nearly 10\% of PDAC cases a hereditary component may be implicated [8, 9].

The pancreas is located behind the stomach along the posterior of the abdomen. The widest part of the pancreas, known as the head, lies in the curve of the duodenum. The body of the pancreas tapers to the left side of the abdomen and the tail ends near the spleen. There are major arteries and sensitive intestinal tissues in close proximity to the pancreas. The hepatic and splenic arteries and the portal and splenic veins comprise the major blood vessels found adjacent to the pancreas. Tumours that occur in the head of the pancreas account for 65\% of pancreatic cancer tumours, while 15\% occur in the body or tail. Ductal adenocarcinoma accounts for 95\% of pancreatic cancers [1]. The proximity of the pancreas to sensitive tissues is one of the challenges that exists in treating pancreatic cancer.
1.1.2 Diagnosis and staging of pancreatic cancer

Few early symptoms are associated with pancreatic cancer, and the symptoms remain vague, such as back pain, lethargy, and new onset diabetes [10]. In addition to being nearly asymptomatic, pancreatic cancer is very aggressive, such that patients are often at an advanced stage in disease progression at the time of initial diagnosis [11]. Less than 20% of pancreatic cancer patients are considered candidates for surgical resection due to tumour invasion of major blood vessels or metastases to the liver or other organs [12]. Pancreatic cancer is staged based on the TNM classification (extent of the tumour, the extent of lymph node involvement, and the presence of metastases) through assessment by endoscopic ultrasound or computed tomography (CT) imaging [13, 14]. Figure 1.1 shows the coronal view of a pancreatic cancer tumour that is resectable (Panel A) and is separate from a major blood vessel, the mesenteric vein, and a tumour (Panel B) that has surrounded two major blood vessels, the celiac artery and the superior mesenteric artery, and therefore has been deemed unresectable [15]. The 5-year survival rates for patients who are treated surgically are 15-20% [1]. Despite up to 20% of pancreatic cancer patients being candidates for the only curative treatment option of surgical resection, the overall 5-year survival rate for pancreatic cancer is low at 6%. Patients who present as having resectable tumours, are sometimes found to have unresectable and locoregionally metastasized tumours upon surgery [16]. The presence of undetectable metastatic disease also contributes to the poor outcome even when patients are staged as having resectable tumours.

1.1.3 Current treatment of pancreatic cancer

The only potentially curative treatment option for pancreatic cancer patients is surgical resection of the tumour. As previously stated, less than 20% of patients presenting with pancreatic cancer are candidates for surgical resection due to locoregional invasion by the tumour or metastatic disease [12]. The surgical protocol used depends on the location of the pancreatic cancer tumour. Pancreatic tumours present in the head of the pancreas are removed by a pancreaticoduodenectomy, also known as the Whipple procedure. The Whipple procedure removes part of the stomach, the pylorus, duodenum, gall bladder, common bile duct, and the head of the pancreas. The remaining part of the pancreas must then be surgically reconnected to the gastrointestinal tract [17]. The mortality rate associated with the Whipple procedure is 3-5% [18].
Figure 1.1. Coronal view of CT images showing the difference between resectable and unresectable pancreatic cancer tumours. Panel A) shows an example of a resectable pancreatic cancer tumour (red arrow) that demonstrates clear separation from the neighboring mesenteric vein (blue arrow). Panel B) depicts an unresectable pancreatic cancer tumour (red arrows) that encompasses the celiac and mesenteric arteries. Reprinted from Wong J [15].
Gemcitabine (GEM) had been the first-line monotherapy for locally advanced pancreatic cancer patients since the late 1990’s when it was shown to be marginally superior to 5-fluorouracil [19]. Recently, albumin bound paclitaxel particles (nab-paclitaxel) was shown to have a synergistic anti-tumour effect when combined with GEM in preclinical mouse studies and this combination is often delivered as first-line treatment in patients with metastatic pancreatic cancer following the results of a phase III clinical trial where this combination demonstrated improved survival rates and progression free survival [20, 21]. Folfirinox, also frequently used as first-line treatment, is a chemotherapy regime involving oxaplatin, irinotecan, leucovorin, and 5-fluorouracil that was shown to improve survival rates compared to GEM (11.1 vs 6.8 months) in metastatic pancreatic cancer patients in a phase II/III clinical [22]. Despite these improvements to the standard chemotherapy options for pancreatic cancer treatment over the last 15 years, the overall 5-year survival rate remains very low at 6%, and so, new treatment options are urgently needed.

The role of radiation therapy in the treatment of pancreatic cancer is highly controversial mainly due to the high small bowel toxicity associated with its delivery [23]. Radiation therapy for pancreatic cancer is evolving from traditional external beam radiation therapy, and most notably, stereotactic body radiation therapy (SBRT) is now being introduced. SBRT has shown promise for treating locally advanced tumours in pancreatic cancer patients in phase I and II clinical trials at a single-fraction dose of 25 Gy, however, these studies showed that high gastrointestinal toxicity was common at this dose [24-27]. Currently, SBRT in combination with folfinox is being studied in phase II and III clinical trials (NCT02128100, NCT02292745, and NCT01926197).

Drugs with the ability to specifically target cancer cells are emerging for the treatment of pancreatic cancer. Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein of the Type 1 human epidermal growth factor receptor (HER/EGFR/ErBb) family, whose activation triggers many signaling pathways leading to cell proliferation, survival, and invasion [28]. EGFR overexpression is present on up to 90% of pancreatic cancer tumours and its overexpression is correlated with a more advanced disease stage, making it attractive as a target for identifying and potentially treating aggressive tumours [28, 29]. Figure 1.2 shows strong EGFR staining of pancreatic cancer cells by immunohistochemistry [30]. EGFR is activated through the binding of various ligands such as epidermal growth factor (EGF), transforming growth factor α (TGFα),
Figure 1.2. Representative cases of EGFR expression in PDAC by immunohistochemistry. Panel A) shows strong membrane score 3+, magnification 40× and Panel B) shows nuclear EGFR staining, magnification 100×. Reprinted with permission from Walsh N [30].
amphiregulin (AREG), epigen, β-cellulin, heparin-binding EGF (HB-EGF), and epiregulin [31, 32]. The binding of ligands to EGFR lead to the phosphorylation of its intracellular tyrosine kinase domain which leads to downstream signal transduction that ultimately lead to proliferation, angiogenesis, as well as invasion and metastasis [33]. Erlotinib is a small molecule tyrosine kinase inhibitor that prevents phosphorylation of the tyrosine kinase domain of EGFR which in turn inhibits extracellular-regulated kinase (ERK), Signal Transducer and Activator of Transcription proteins (STAT), and phosphatidylinositol 3-kinase and protein kinase B (PI3K-AKT) signaling [34]. Erlotinib, in combination with GEM, remains the only Health Canada approved targeted therapy for the treatment of unresectable pancreatic cancer [35]. Other inhibitors of EGFR include the monoclonal antibodies (mAb) cetuximab and panitumumab which bind to the extracellular domain to inhibit the binding of EGFR ligands [36]. However, in a phase III clinical trial, cetuximab failed to improve the outcome for patients with pancreatic cancer compared to GEM alone [37]. The action of both cetuximab and panitumumab have been shown to require the presence of wild-type Kras, a gene downstream of EGFR responsible for controlling cell proliferation, as shown in Figure 1.3 [38, 39]. Unfortunately, the majority of pancreatic cancer tumours have mutated Kras that results in its constitutive activation and refractoriness to upstream EGFR blocking, and so therapy with these naked mAbs cannot be effective [40]. The molecular mechanisms that allow erlotinib, and not anti-EGFR mAbs to be effective in the presence of Kras mutated pancreatic cancer remains unclear [41]. Despite ineffectiveness of naked mAbs for Kras mutant pancreatic cancers, using these mAbs with radionuclides conjugated can deliver cytotoxic ionizing radiation regardless of Kras mutations, as will be discussed further in section 1.2.
Figure 1.3. Diagram of the EGFR signaling pathway. EGFR ligands bind to EGFR and induce dimerization. EGFR has a tyrosine kinase domain which is phosphorylated after dimerization and causes the activation of EGFR. Activated EGFR stimulates the downstream signal transduction. As a result, proliferation, angiogenesis, invasion, and metastasis are increased while apoptosis is reduced. Cetuximab and panitumumab are mAbs that target the binding site of EGFR and inhibit dimerization. Erlotinib is a small molecule that inhibits phosphorylation of tyrosine domains of EGFR. Mutations of the Kras gene have been shown to render anti-EGFR mAbs ineffective for therapy of pancreatic cancer, however, using these mAbs with radioactivity conjugated can deliver cytotoxic ionizing radiation regardless of Kras mutations.
1.2 Radioimmunoconjugates for molecular imaging and therapy of pancreatic cancer

The role of radiation therapy in the treatment of pancreatic cancer is highly controversial mainly due to the high small bowel toxicity associated with its delivery [23]. One potential strategy for harnessing radiation for effective treatment of pancreatic cancer lies in the use of radioisotopes conjugated to monoclonal antibodies (mAbs) which can target receptors overexpressed on pancreatic cancer tumours, thereby specifically delivering ionizing radiation to tumours [42]. These radiolabeled mAbs are known as radioimmunoconjugates (RICs) and can be used for radioimmunotherapy (RIT) as well as for molecular imaging. RIT could be useful as an adjuvant therapy following surgical resection of pancreatic cancer tumours to target residual disease remaining following surgery as well as undetectable metastatic disease. This may prevent local recurrence and progression to metastatic pancreatic cancer, when used in combination with other treatments such as chemotherapy. RIT could also provide a ‘boost’ to conventional radiation therapy for treatment of locally advanced pancreatic cancer. Conventional radiation therapy can cause high toxicity to abdominal organs neighbouring the pancreas, and the addition of RIT to the treatment regime may enhance radiation delivery locally to pancreatic tumour cells, potentially without contributing significantly to the toxicity to neighbouring normal organs. This is especially true because the β-particles most often used for RIT have a short range of only 2-10 mm, providing a highly conformal radiation delivery to tumours that are targeted by the radiolabeled mAbs.

Radionuclides that are commonly conjugated to mAbs or peptides for use in RIT include alpha (α)-emitters such as $^{211}$At, $^{213}$Bi, and $^{225}$Ac; beta (β)-emitters such as $^{64}$Cu, $^{177}$Lu, $^{131}$I, and $^{90}$Y; and Auger electron emitters such as $^{111}$In, $^{123}$I, $^{125}$I, $^{99m}$Tc and $^{67}$Ga [43]. A characteristic of consideration when selecting a radionuclide for RIT or nuclear imaging is the linear energy transfer (LET). LET describes the energy deposited over the track length of the radiation in tissues. Figure 1.4 illustrates the different distances that β-particles, α-particles, and Auger electrons travel in tissues. The low LET of β-particles, 0.1–1.0 keV/μm, results in relatively low deposition of energy in tumour cells which may limit the potency of their cytotoxic effects. In contrast, α-particles have LET values of 50–230 keV/μm and Auger electrons have LET of 4 to 26 keV/μm, which would render these forms of radiation more cytotoxic than β-emitters.
Figure 1.4. Illustration of the track of α-particles, β-particles or Auger electrons emitted by radiolabeled monoclonal antibodies 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 [44].
Moreover, α-particles have a range of only 50–100 μm, which restricts their cross-fire effect to 5–10 cell diameters, in contrast to β-particles which irradiate up to 200–1000 cell diameters from the site of decay (Figure 1.4). The subcellular nanometer range of Auger electrons eliminates the cross-fire effect and requires that these electrons be released in close proximity to nuclear DNA to cause lethal double-strand breaks (DSBs). The short range of α-particle and Auger electron-emitting radionuclides 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 makes this form of radiation more feasible for treating larger tumour masses (>1 cm) [45].

1.2.1 Radionuclides emitting β-particles for RIT

In β-decay, an unstable atomic nucleus that has an excess of neutrons converts one neutron into a proton by emitting a β-particle and an antineutrino (Figure 1.5). The range of β-particles in tissue is much longer than for α-particles or Auger electrons (Figure 1.4) [44]. β-particles transfer their energy to the surrounding matter over a distance of several millimeters and deposit most of their energy at the end of their track length (Bragg peak) and are considered to be low LET radiation. Low LET radiation is dependent on oxygen to generate oxygen free radicals, as such, a hypoxic state will decrease the effectiveness of β-emissions [46]. The range of β-particles makes them most suitable for treating tumours with a diameter of 2-12 mm (200-1200 cell diameters) since for smaller tumours most of the radiation energy would be deposited outside the target volume due to the Bragg peak phenomenon [47]. Table 1.1 highlights common β-emitters and their most relevant characteristics. The long range of β-particles can be beneficial when considering tumour heterogeneity since in this case RICs targeted to tumour cells may deposit their cytotoxic radioactivity in neighbouring non-targeted cells that lie within striking distance of the β-particles, in a phenomenon known as the crossfire effect [47, 48]. Most clinical trials of RIT have employed the radionuclides 131I and 90Y, which are both β-emitters, and represent the current standard to which all other radionuclides are compared [48-50]. Many of published clinical trials of RIT have demonstrated the efficacy of 131I and 90Y, particularly for the treatment of haematological and in some cases for solid malignancies [48].
Figure 1.5. Diagram of β-decay. In β-decay, an unstable atomic nucleus that has an excess of neutrons converts one neutron (n) into a proton (p) by emitting a β-particle (e\textsuperscript{-}) and an antineutrino (\overline{\nu}_e). Author: By Inductiveload [Public domain], from Wikimedia Commons (https://commons.wikimedia.org/wiki/File:Beta-minus_Decay.svg from Wikimedia Commons).
### Table 1.1. Common radionuclides emitting β-particles for conjugation to biomolecules for RIT of cancer with characteristics of β-emissions highlighted despite the occurrence of other emissions.

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Half-life ( (t_{1/2p}) )</th>
<th>Particle(s) Emitter</th>
<th>Particulate Energy</th>
<th>Range in Tissue</th>
<th>Chelation Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{90}\text{Y})</td>
<td>2.7 days</td>
<td>β</td>
<td>2.3 MeV</td>
<td>12 mm</td>
<td>DOTA</td>
</tr>
<tr>
<td>(^{64}\text{Cu})</td>
<td>12.7 h</td>
<td>β, Auger</td>
<td>578 keV</td>
<td>2 mm</td>
<td>NOTA, DOTA</td>
</tr>
<tr>
<td>(^{67}\text{Cu})</td>
<td>2.58 days</td>
<td>β,γ</td>
<td>390/482/575 keV</td>
<td>0.2 mm</td>
<td>NOTA, DOTA</td>
</tr>
<tr>
<td>(^{177}\text{Lu})</td>
<td>6.7 days</td>
<td>β,γ</td>
<td>498 keV</td>
<td>2 mm</td>
<td>NOTA, DOTA, CHX-DTPA</td>
</tr>
<tr>
<td>(^{131}\text{I})</td>
<td>8.1 days</td>
<td>β,γ</td>
<td>0.6 MeV</td>
<td>2 mm</td>
<td>other(^a)</td>
</tr>
<tr>
<td>(^{188}\text{Re})</td>
<td>17 h</td>
<td>β</td>
<td>1.1 MeV</td>
<td>3 mm</td>
<td>other(^b)</td>
</tr>
</tbody>
</table>

\(^a\)Direct radioiodination with chloramine-T or Iodogen; indirect conjugation with N-succinimidyl ester of 3-(tri-n-butylstannyl) benzoate (ATE), N-succinimidyl 5-[\(^{131}\text{I}\)]iodo-3-pyridinecarboxylate (SIPC), N-succinimidyl 4- guanidinomethyl-3-[\(^{131}\text{I}\)]iodobenzoate (SGMIB).

\(^b\)Binding to thiols; chelation by tetradeutate complexes; HyNic; interaction of carbonyl complex with histidine residues; trisuccin.
Some β-emitting radionuclides emit other forms of radiation that can be used for nuclear imaging. Figure 1.6 shows two such radionuclides, $^{64}\text{Cu}$ and $^{177}\text{Lu}$. $^{64}\text{Cu}$ emits β-particles ($\beta^- = 570 \text{ keV (39.0\%)}$) and Auger electrons that can be useful for RIT, as well as positrons ($\beta^+ = 650 \text{ keV (17.4\%)}$) that allow PET in addition to having therapeutic potential for “positherapy”. $^{177}\text{Lu}$ (half-life 6.7 d) emits moderate energy β-emission ($\beta^- = 498 \text{ keV (78.6\%)}, \beta^- = 385 \text{ keV (9.1\%)}, \text{and } \beta^- = 176 \text{ keV (12.2\%)}$) as well as low abundance γ-emissions ($\gamma = 113 \text{ keV (6.4\%) and } \gamma = 208 \text{ keV (11\%)}$) that allow SPECT. This concept combining diagnostics with therapeutics from a single agent is termed “Theranostics”. The development of a theranostic agent that could provide sensitive pancreatic cancer imaging of metastatic disease as well as characterizing the receptor expression of these lesions for subsequent RIT is appealing; PET and SPECT imaging modalities will be discussed further in section 1.3. To conjugate $^{64}\text{Cu}$ to a targeting moiety, such as a mAb, the mAb must first be modified with an appropriate chelator. For many years tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) was used for $^{64}\text{Cu}$ chelation, however, this led to sustained liver and kidney accumulation of $^{64}\text{Cu}$, and in recent years 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) has become known to have greater stability in complexing $^{64}\text{Cu}$ than DOTA [51, 52]. $^{177}\text{Lu}$ radiolabeling is commonly performed with DOTA and has shown excellent in vivo stability in preclinical and clinical studies [53-56]. NOTA has also been successful in labeling $^{177}\text{Lu}$ when conjugated with panitumumab where a radiochemical purity maintained <90% 7 days following labeling, without interfering with EGFR binding activity of panitumumab [55]. The potential of $^{64}\text{Cu}$-labeled anti-tumour mAbs or peptides for RIT of malignancies has been demonstrated by several investigators [57-60] and the utility of this radionuclide for PET imaging has been demonstrated in phase I clinical trials where $^{64}\text{Cu}$-labeled diacetyl-bis(N-methylthiosemicarbazone), ($^{64}\text{Cu}$-ATSM), was used to image hypoxia in cancer of the uterine cervix [61].

1.2.2 Radionuclides emitting α-particles for RIT

An α-particle is a helium-4 ($^4\text{He}$) nucleus consisting of two protons and two neutrons which carries a +2 charge (Figure 1.7A). α-Particles have much higher LET (50–230 keV/μm) than β-particles emitted by radionuclides such as $^{131}\text{I}, ^{90}\text{Y}, ^{177}\text{Lu}, ^{186}\text{Re}$ or $^{188}\text{Re}$ (LET=0.1–1.0 keV/μm) due to their short range in tissues (28–100 μm; Figure 1.4) [62]. Table 1.2 highlights common α-emitters for RIT and their most relevant characteristics. The range of α-particles in tissues corresponds to only 5–10 cell diameters, which restricts the deposition of radiation to the
Figure 1.6. Decay schemes for $^{64}$Cu and $^{177}$Lu indicating the abundance and energy of emissions and the applications for the different types of radiation emitted.
**Figure 1.7.** Diagram of α-emissions and Auger electrons. (A) α-particles are high energy (several MeV) radiation composed of two protons and two neutrons and carry a 2+ charge. (B) Auger electrons are very low energy (<25 keV) electrons with a 1-charge emitted by radionuclides that decay by electron capture (EC). In EC, a proton in the nucleus captures an electron from an inner orbital shell converting the proton to a neutron. This creates a vacancy in the shell which is filled by an electron from a higher shell, creating a subsequent vacancy, which is then filled by decay of an electron from a higher shell etc. Ultimately, the energy released from these electron transitions is imparted on an outer orbital electron which is ejected from the atom (Auger electron), creating a transiently positively-charged nucleus. Reprinted with permission from Aghevlian S [44].
Table 1.2. Common radionuclides emitting α-particles for conjugation to biomolecules for RIT of cancer with characteristics of α-emissions highlighted despite the occurrence of other emissions. The range in tissue for all α-emitters is 28-100 μM [44].

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Half-life (t₁/₂p)</th>
<th>Particulate Energy</th>
<th>Chelation Methodᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{225}$Ac</td>
<td>10 days</td>
<td>5.8 MeV</td>
<td>DOTA or NETA</td>
</tr>
<tr>
<td>$^{211}$At</td>
<td>7.2 h</td>
<td>7.4 MeV</td>
<td>Radioastatination</td>
</tr>
<tr>
<td>$^{212}$Bi</td>
<td>61 min</td>
<td>6.0 MeV</td>
<td>DTPA</td>
</tr>
<tr>
<td>$^{213}$Bi</td>
<td>46 min</td>
<td>6-8.4 MeV</td>
<td>DTPA</td>
</tr>
<tr>
<td>$^{212}$Pb</td>
<td>10.6 h</td>
<td>6.0 MeV</td>
<td>TCMC</td>
</tr>
<tr>
<td>$^{227}$Th</td>
<td>18.7 days</td>
<td>5.9 MeV</td>
<td>DOTA</td>
</tr>
</tbody>
</table>

ᵃDOTA: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; DTPA: diethylenetriaminepentaacetic acid; NETA: 4-[2-(bis-carboxymethyl-amino)-ethyl]-7-carboxymethyl-[1,4,7]triazonan-1-yl-acetic acid; TCMC: 2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraaza-1, 4, 7, 10-tetra-(2-carbamoyl methyl)-cyclododecane.
targeted cell and closely neighbouring cells. In contrast, β-particles deposit their energy along a track that greatly exceeds the diameter of a single targeted cell [63, 64]. The high LET and short range of α-particles make this form of radiation useful for eradicating circulating malignant cells (e.g. leukemia cells in the blood or bone marrow) or small clusters of cells (e.g. disseminated disease and micrometastases from solid tumours). Indeed, it has been shown that α-emitters are more effective than β-emitters for RIT of small lung metastases in rats, providing greater relative biological effectiveness (RBE) [65, 66]. RBE describes the effectiveness of a particular type of radiation for causing an effect (e.g. cytotoxicity) compared to X- or γ-radiation. A high RBE is desirable for RIT. The decay scheme of some α-emitters (e.g. ²²⁵Ac and ²²⁴Ra) yields daughter products that are α-emitters or β-emitters, and such radionuclides have been proposed as α-particle "radionuclide generator systems" that could deliver a higher radiation absorbed dose to tumours than radionuclides that emit only single α-particles or decay to stable elements or daughter products that emit radiation with lower RBE (e.g. β-particles, X- or γ-radiation) [67].

Although there is great potential with the radionuclide generator concept, a limitation is that the daughter α-emitters in most cases will not remain complexed to chelators substituted onto the mAb since they represent elements with different chemistry, this is true for other radionuclides, but the problem arises with α-emitters in that their daughter products are radionuclides rather than stable, non-radioactive nuclides. In addition, the high recoil energy upon decay also promotes their release from the chelators. Release of daughter radionuclides and their redistribution to normal tissues have been reported for the α-emitter ²²⁵Ac which decays to several daughter radionuclides, including ²¹³Bi, which is also an α-emitter. Redistribution of ²¹³Bi released from ²²⁵Ac-labeled anti-CD33 mAb HuM195 deposited α-radiation in the kidneys causing acute renal dysfunction in nonhuman primates [68, 69]. This phenomenon has not been confirmed in humans however, since early results of a phase I clinical trial of ²²⁵Ac-labeled HuM195 (ClinicalTrials.gov identifier NCT00672165) in patients with acute myeloid leukemia (AML) presented at the annual meeting of the American Society of Clinical Oncology (ASCO) in 2015 indicated that the maximum tolerated dose (MTD) was not yet reached [70].

The RBE of α-particles ranges from 3 to 7, indicating that for the same biological effect a lower dose is required compared to β-radiation which has an RBE of 1 [71]. The primary target of α-particles in causing cell death is considered to be nuclear DNA, and is manifested by DNA DSBs. Studies have shown that when the track of an α-particle traverses the cytoplasm, there
fails to be a lethal effect on a cell, while a track that crosses the nucleus is correlated with cytotoxicity [72]. A single track of ionizing radiation from an α-emitter induces clusters of DNA DSBs, which can be detected in vitro by immunofluorescence probing for phosphorylated histone 2A (γ-H2AX) which accumulates at sites of unrepaired DNA DSBs [63]. Importantly, the induction of DSBs by α-particles is independent of tissue oxygenation, which is advantageous compared to β-emitters for RIT which rely indirect damage to DNA when an electron interacts with, for example, a water molecule to produce a hydroxyl radical (OH-) which in turn damages the DNA [73]. Hypoxia is a common resistance mechanism to treatment of cancer with low LET radiation [75]. High LET radiation such as α-particles directly cleaves the DNA duplex, which does not rely on tumour oxygenation. Wulbrand et al. found that 213Bi-labeled matuzumab, killed hypoxic CAL 33 squamous cell carcinoma cells as effectively as normoxic cells in vitro [74]. The mechanism of cell death from RIT with α-emitters was examined in one report in which intraperitoneally (i.p.) injected 212Pb-labeled anti-human epidermal growth factor receptor-2 (HER2) mAb, trastuzumab, was compared to nonspecific 212Pb-human IgG in mice with disseminated i.p. LS-174T human colon cancer tumours [76]. Ex vivo examination of these tumours revealed that these HER2-targeted radioimmunoconjugates (RICs) caused increased apoptosis and DNA DSBs, delayed DNA damage repair, and caused G2-M cell cycle arrest compared to treatment with control irrelevant RICs. It is important to consider the effects of α-particle RIT on toxicity to normal organs, especially for 211At-labeled mAbs which because 211At is a halogen, may undergo de-astatination in vivo. Free 211At accumulates in the thyroid unless blocked by potassium perchlorate administration [77]. In a phase I clinical trial in 9 patients with ovarian cancer administered 211At-MX35 F(ab’)2 fragments recognizing sodium-dependent phosphate transport protein 2b (NaPi2b), therapeutic radiation absorbed doses were deposited in tumours without toxicity to normal tissues, including the thyroid, by co-administering RIT with potassium perchlorate [78].

1.2.3 Radionuclides emitting Auger electrons for RIT

Radionuclides that decay by electron capture (EC) and/or internal conversion (IC), processes that introduce vacancies in their inner electronic shells of the daughter atoms (Figure 1.7B), emit very low energy (<25 keV) electrons known as Auger electrons. Pierre Auger first discovered these electrons in 1925 [79]. The vacancies created by EC and/or IC are filled by electrons decaying from higher shells creating subsequent vacancies that move progressively towards the outer
shells. These transitions are accompanied by the emission of either a characteristic X-ray or low-energy Auger or IC electron [80]. Unlike Auger electrons, IC electrons are not associated with the emission of an X-ray but rather the difference in energy between two electronic shells is transferred to an outer shell electron that is ejected by the atom. An estimated 5 to 50 electrons of different energies are emitted following every EC and/or IC event and because of their low energies, these electrons have a subcellular, nanometer range (<0.5 μm). Due to their very short path length, Auger electrons have high LET (4 to 26 keV/μm) despite their very low energies, which has the potential to cause multiple lethal DNA DSBs in cancer cells if these electrons are released in close proximity to nuclear DNA [81]. Table 1.3 highlights common Auger electron emitters and their most relevant characteristics.

Since Auger electrons have only nanometer range, their lethal DNA damaging effects are typically dependent on the position of radionuclide decay relative to the DNA within the nucleus [80]. This was demonstrated in the literature when the Auger electron emitters $^{123}$I, and $^{125}$I conjugated to iododeoxyuridine (I UdR) which is incorporated directly into DNA significantly decreased mammalian cell survival [82, 83]. In these studies, the mean lethal dose of radiation for $^{125}$I-I UdR was 7-fold lower than X-radiation for the same degree of cell killing, indicating a high RBE value for the Auger electrons emitted by $^{125}$I [82]. On the contrary, no major enhancement of cytotoxic effects compared to low LET $\gamma$-radiation was found for the Auger electron-emitters $^{51}$Cr, $^{67}$Ga, $^{75}$Se, $^{125}$I, or $^{201}$Tl localized in the cytoplasm [84-87], bound to the cell membrane [87, 88] or located outside the cell. Decay of an Auger electron-emitter in close proximity to nuclear DNA results in a high RBE and fully exploits the high LET of these electrons for killing cells [84-87]. Decay of an Auger electron-emitting radionuclide near but not within the DNA duplex can efficiently generate single-strand breaks (SSB) and DSBs which are caused indirectly by hydroxyl radical (•OH)-mediated ionizations [89, 90]. Since the most lethal DNA damaging effects of Auger electrons are dependent on their proximity to nuclear DNA, studies have exploited nuclear translocation sequence (NLS) peptides to transport mAbs labeled with the Auger electron-emitter, $^{111}$In, to the cell nucleus following receptor-mediated internalization by cancer cells [91-93]. These 13-mer peptides (CGYGPKKKRKVGG) harbor the NLS of SV-40 large T-antigen [underlined] which interacts with the importin-α/β machinery to transport the mAbs across the nuclear membrane [94]. It stand to reason that since the cell membrane has a critical function in cell survival, the effects of Auger electrons emitted by
Table 1.3. Common radionuclides emitting Auger electrons for conjugation to biomolecules for RIT of cancer with characteristics of Auger electrons highlighted despite the occurrence of other emissions. The range of Auger electrons in tissues is <0.5 µm [44].

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Half-life (t_{1/2p})</th>
<th>Total electron energy (keV)</th>
<th>Chelation Method^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>^{125}\text{I}</td>
<td>57 days</td>
<td>19.4</td>
<td>Radioiodination labeling</td>
</tr>
<tr>
<td>^{123}\text{I}</td>
<td>13.2 h</td>
<td>27.6</td>
<td>Radioiodination labeling</td>
</tr>
<tr>
<td>^{111}\text{In}</td>
<td>2.8 days</td>
<td>32.7</td>
<td>DTPA or DOTA</td>
</tr>
<tr>
<td>^{67}\text{Ga}</td>
<td>3.3 days</td>
<td>34.4</td>
<td>DFO or DOTA</td>
</tr>
<tr>
<td>^{99m}\text{Tc}</td>
<td>6.0 h</td>
<td>16.3</td>
<td>HyNic or N\textsubscript{2}S\textsubscript{2} chelator</td>
</tr>
<tr>
<td>^{64}\text{Cu}</td>
<td>12.7 h</td>
<td>18.2</td>
<td>NOTA or DOTA</td>
</tr>
</tbody>
</table>

^a\ DTPA: diethylenetriaminepentaacetic acid; DOTA: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; DFO: desferrioxamine; HyNic: hydrazinenicotinamie; N\textsubscript{2}S\textsubscript{2}: diamide dimercaptide; NOTA: 1, 4, 7-triazacyclononane-N,N',N''-triacetic acid.
membrane-bound radiolabeled mAbs should also to be considered [95]. The need for internalization of mAbs for Auger electron RIT has been questioned by recent studies that compared the cytotoxicity of radioactivity in the nucleus, at the cell surface, and in the cytoplasm of A431 eidermoid cancer cells or SK-OV-3 ovarian cancer cells. In this study, cells were exposed to internalizing 125I-labeled anti-EGFR 225 or anti-HER2 trastuzumab mAbs or non-internalizing anti-carcinoembryonic antigen (CEA) 35A7mAbs or 125I-labeled tat peptides, which penetrate cells and are transported to the cell nucleus [95]. Nuclear 125I was the most cytotoxic due to the DNA damaging properties of the Auger electrons, yet, targeting the cell membrane proved more effective for killing cells than 125I deposited in the cytoplasm [95]. Due to the small range in tissues, Auger electrons do not have a “cross-fire” effect that could irradiate and kill neighbouring cells that are not targeted by the radiolabeled mAbs [96]. This is advantageous in minimizing the normal tissue toxicity, particularly when considering hematopoietic stem cells in the bone marrow, which is dose-limiting for mAbs labeled with β-emitters [48]. The use of Auger electron RIT for solid tumours may be limited by poor tumour penetration of radiolabeled mAbs due to their short range in tissues [97]. Despite this supposed limitation, Auger electron-emitters exhibit a localized “bystander effect” which promotes the apoptosis of non-targeted tumour cells near the targeted cells [98-100]. The bystander effect may be either tumour growth inhibitory, or tumour growth stimulatory, as has been observed in some cases. For example, in male Ncr nude mice, co-inoculation of unlabeled LS174T human colon cancer cells with 125I-labeled cells demonstrated significant tumour growth inhibition compared to inoculation of only unlabeled cells, by contrast, a mixture of unlabeled and 123I-labeled cells exhibited enhanced tumour growth compared to inoculation of only unlabeled cells [100]. The bystander effect may contribute to the effectiveness of Auger RIT for cancer treatment.

1.2.4 Targets for molecular imaging and RIT of pancreatic cancer

Potential targets for RIT of pancreatic cancer include EGFR and HER2, both members of the human epidermal growth factor receptor (HER/EGFR/ErbB) family, the cell surface glycoprotein mucin-1 (MUC1), integrin α6β4, and the carbohydrate antigen sialyl Lewis a (CA19-9), all of which are overexpressed on pancreatic cancer cells to varying degrees. EGFR is overexpressed on up to 90% of pancreatic cancer tumours [29] and has been a target for RIT in preclinical pancreatic cancer studies using the α-emitter 213Bi, and the β-emitter 177Lu [74, 101]. HER2 is overexpressed in 60% of pancreatic cancers [102] and is currently being studied
clinically as a potential target for RIT in a phase II clinical trial investigating the alpha-emitter $^{212}$Pb conjugated to trastuzumab as a mono-RIT in patients with HER2+ malignancies including pancreatic cancer (ClinicalTrials.gov Identifier: NCT01384253). MUC1 has been shown to be overexpressed in 55-95% of pancreatic cancer patients [103, 104] and has been targeted for RIT preclinically with $^{131}$I labeled mAb MAb-PAM4, and clinically with $^{90}$Y-clivatuzumab [105, 106]. MUC1 was studied clinically as a target for RIT of pancreatic cancer with $^{90}$Y-clivatuzumab. In the first phase I trial the maximum tolerated dose (MTD) of single-dose $^{90}$Y-clivatuzumab was determined to be 20 mCi/m$^2$ [106]. In a second phase I trial fractionated doses of $^{90}$Y-clivatuzumab were administered in combination with low-dose GEM, with a reduced MTD for $^{90}$Y-clivatuzumab of 12 mCi/m$^2$; this enhanced the outcome to therapy where 6 patients (16%) had partial responses according to CT-based Response Evaluation Criteria in Solid Tumours, and 16 patients (42%) had stabilization as their best response (58% disease control) [107]. Indeed, FDG-PET imaging revealed a decrease in tumour size in one patient 4 weeks after treatment [107]. This RIT agent was also studied in phase III but was terminated when patients receiving GEM plus $^{90}$Y-clivatuzumab did not experience improved survival over GEM plus a placebo (ClinicalTrials.gov Identifier: NCT01956812) though the treatment was well tolerated in phase I and II clinical trials [106]. It is worthwhile to note that in clinical trials of new treatments for pancreatic cancer it is very difficult to demonstrate improved survival due to patients being in late stage of disease progression and the fact that pancreatic cancer is very aggressive, limiting the follow-up period for assessment of effectiveness. Another target for RIT of pancreatic cancer in preclinical studies is integrin α6β4 which has been demonstrated as being overexpressed in pancreatic cancer [108]. Recently, $^{90}$Y-labeled to the anti-integrin α6β4 mAb, ITGA6B4, has shown promise in pancreatic cancer xenograft mouse models [109]. A phase I clinical trial is currently recruiting pancreatic cancer patients with CA19-9 overexpression for a dose escalation study for treatment with $^{177}$Lu-labeled human mAb 5B1 (MVT-107), NCT03118349.

EGFR is an ideal target for RIT of pancreatic cancer since this has been shown to be present in up to 90% of pancreatic cancer tumours [29]. However, as mentioned in section 1.1.3, targeting EGFR with mAbs for pancreatic cancer treatment is not effective in the presence of the Kras mutation, which is present in up the majority of pancreatic cancer [40]. This is possibly why clinical trials have yet to explore RIT targeting EGFR for pancreatic cancer. Yet, targeting EGFR for RIT does not rely on inhibiting downstream signaling pathways, but rather the expression of
the target to deliver cytotoxic ionizing radiation, and so, the presence of the Kras mutation is not an obstacle in targeting EGFR for RIT. Al-Ejeh \textit{et al.} demonstrated that RIT with a $^{177}$Lu-labeled anti-EGFR mAb combined with radiosensitizing agents caused tumour growth arrest in mice engrafted with PANC-1 pancreatic cancer xenografts \cite{101}. Since pancreatic cancer is an aggressive disease with poor prognosis, it is likely that RIT would be enhanced dramatically with radiosensitizing agents for the treatment of pancreatic cancer, as will be discussed in section 1.7. With particular significance to Auger electrons for RIT of cancer, EGFR contains a putative NLS (RRHIVRKRTLLR), which is found in the transmembrane domain of the receptor \cite{110}. Nuclear transport of EGFR would allow the short range of Auger electrons to be effective in causing DNA DSBs. This potential has been shown in the literature where $^{111}$In-labeled nimotuzumab, a mAb against EGFR, modified with an NLS caused a 2-fold increase in DNA DSBs compared to $^{111}$In-nimotuzumab, as was assessed by immunofluorescence of $\gamma$-H2AX foci in the nucleus \cite{111}. With EGFR-targeted therapy, normal tissue toxicity is a point of consideration since EGFR expression is found in the liver, kidneys, and intestinal tissues \cite{112-114}. Nevertheless, mAbs targeting EGFR have been employed in clinical practice for cancer treatment \cite{115}. Indeed, radiolabeled panitumumab has been studied as a PET imaging agent in patients with metastatic colon cancer who were injected with 37 MBq of $^{89}$Zr-panitumumab where no significant toxicity was observed \cite{116}.

1.2.5 Antibody forms for molecular imaging and RIT

Radionuclides can be conjugated to mAbs as intact immunoglobulins, the most common type being immunoglobulin G (IgG), or as smaller antibody forms including F(\text{ab}')$_2$ fragments, Fab fragments, which are prepared from enzymatic digestion of intact IgGs, and engineered antibody fragments including single chain variable fragments (scFv), diabodies, minibodies, and affibodies. IgGs are comprised of heavy chains and light chains. The amino-terminal variable or V domains of the heavy and light chains (V$_H$ and V$_L$, respectively) make up the V region of the antibody and account for the ability to bind specific antigens, while the constant domains, C domains, of the heavy and light chains (C$_H$ and C$_L$, respectively) make up the C region, as shown in Figure 1.8. The multiple heavy-chain C domains are numbered from the amino-terminal end to the carboxy terminus, for example C$_H$1, C$_H$2, and so on. Figure 1.8 shows the different antibody forms of intact IgG antibodies (150 kDa), and enzymatically produced F(\text{ab}')$_2$ fragments (110 kDa), and Fab fragments (50 kDa). F(\text{ab}')$_2$ and Fab fragments can be generated
Figure 1.8. Structures of intact IgG, F(ab')2 fragments, and Fab fragments.
by enzymatic digestion of IgGs with pepsin and papain, respectively. There are several reasons that mAb fragments are preferable over intact IgGs. One advantage of using antibody fragments is that the Fc domain, which is comprised of the C_{12} and C_{13} domains illustrated in Figure 1.8, is cleaved from the structure upon enzymatic digestion. The absence of the Fc domain eliminates the binding that occurs between Fc regions and the Fc receptors of non-targeted cells [117]. Additionally, F(ab')_2 fragments differ in their pharmacokinetic characteristics compared to intact IgGs resulting in distinct blood clearance and tumour localization patterns, clearing faster from the circulation than intact IgGs while demonstrating better penetration into tumour sites [118-123]. When considering the application of RICs for molecular imaging to monitor tumour response to therapy, detection of metastatic disease, and dosimetric calculations, the rapid blood clearance of mAb fragments result in higher signal-to-noise ratios occurring at earlier time points, as well as tumour/blood (T/B) ratios, which allows shorter lived radioisotopes, such as ^{68}\text{Ga} (half-life = 68 min) to be used. This ultimately reduces the normal tissue burden of RICs in patients. Another benefit of the rapid clearance of mAb fragments is that there is less potential for an immunogenic response in patients which would allow repeated treatment with RICs [124].

Smith-Jones et al. demonstrated faster blood clearance and higher T/B ratios for ^{111}\text{In}-trastuzumab F(ab')_2 compared to the intact IgG at 24 h p.i. in BT-474 HER2-overexpressing human BC xenografts in nude athymic mice (10.0 vs. 3.4, respectively) [125]. In this study, high contrast PET images of BT-474 tumours in mice were achieved at only 3 h after administration of ^{68}\text{Ga}-trastuzumab F(ab')_2 fragments. The faster tumour accumulation of F(ab')_2 fragments compared to intact IgG is seen in the literature in LS-174T human colorectal xenografts in athymic mice, where in one study examining the biodistribution and blood pharmacokinetics of ^{111}\text{In}-panitumumab F(ab')_2 fragments the authors report the tumour accumulation at 24 h p.i. as 21.42 ± 7.67 %ID/g [126], approximately double that of intact panitumumab reported at 24 h p.i., 13.27 ± 8.40 %ID/g [127]. Comparing these studies also shows the faster blood clearance of F(ab')_2 fragments compared to intact IgG where the blood compartment for ^{111}\text{In}-panitumumab F(ab')_2 fragments was found to be 6.84 ± 2.30 %ID/g at 24 h p.i., approximately one-half of that reported for intact panitumumab, 11.69 ± 1.47 %ID/g [126, 127]. In these studies, the panitumumab F(ab')_2 fragments exhibited faster tumour accumulation compared to intact IgG at 24 h p.i., however, a higher tumour accumulation of intact ^{111}\text{In}-panitumumab was observed at a later time point, 44.08 ± 9.53 %ID/g at 48 h. The higher tumour accumulation of the intact IgG
may be an indication that this form of the mAb would be preferable for RIT, however, with the increased risk of causing an immune response in a patient, antibody fragments may still be preferable.

1.2.6 Amplifying radiation delivered by RICs with metal chelating polymers

Antibody-conjugated metal chelating polymers (MCPs) that present multiple chelators for complexing radiometals can significantly increase the specific activity (MBq per µg mAb) of RICs [128, 129]. For example, in a study examining $^{111}$In-labeled NLS-trastuzumab monosubstituted with DTPA, only one in 50 molecules was radiolabeled with a specific activity of <0.24 MBq/µg (<3.6 MBq/µmol) being achieved [93]. There is a risk in increasing the number of chelators in that the immunoreactivity of the mAb binding site for its target may decrease [130]. Incorporating MCPs into RICs can be useful in amplifying the radioactivity delivered to a cancer cell per mAb bound, thereby potentially increasing sensitivity for radioimaging, or increasing the effectiveness in RIT, without compromising the immunoreactivity of the mAb.

A major consideration in the composition of an MCP is their backbone. One study compared three structurally diverse MCPs which had been biotinylated for linkage to a streptavidin-modified trastuzumab Fab fragment to exploit the high affinity, non-covalent binding between biotin and streptavidin [131]. These MCPs, pictured in Figure 1.9, were structurally diverse: Bi-PAm(DTPA)$_{40}$ was comprised of a polyacrylamide backbone harbouring 40 pendant DTPA groups; Bi-PAsp(DTPA)$_{33}$ contained a polyaspartamide backbone with 33 pendant DTPA groups; and Bi-PGlu(DTPA)$_{28}$ contained a polyglutamide backbone with 28 DTPA groups. In this study, the authors found that saturation of the DTPA chelators with a non-radioactive metal was required to reduce the negative charge of the molecule that occurred due to un-chelated DTPA groups. Upon saturation of the DTPA chelators Bi-PAsp(DTPA)$_{33}$ and Bi-PGlu(DTPA)$_{28}$ became zwitterionic, with a net neutral charge, while Bi-PAm(DTPA)$_{40}$ continued to carry a net negative charge. The most notable result was that Bi-PAm(DTPA)$_{40}$ had the highest kidney and liver accumulation at 24 p.i., 34.8 ± 2.2 %ID/g and 35.2 ± 1.9 %ID/g, respectively. This study concluded that zwitterionic MCPs composed of a polyaspartamide backbone exhibited the most desirable tumour and normal tissue distribution properties [131]. In a subsequent study, another biotinylated MCP with a polyacrylamide backbone and 40 DTPA groups was studied with
Figure 1.9. Chemical structures of metal-chelating polymers (MCPs) with a terminal biotin functionality. Bi-PAm(DTPA)$_{40}$ has a polyacrylamide backbone harbouring 40 pendant DTPA groups. Bi-Pasp(DTPA)$_{33}$ has a polyaspartamide backbone harbouring 33 DTPA groups. Bi-PGlu(DTPA)$_{28}$ has a polyglutamide backbone harbouring 28 DTPA groups. R represents biotin and the structure of DTPA is shown. Reprinted with permission from Boyle AJ [131].
addition of ethylenediamine (EDA) to the pendant group that allowed the molecule to become zwitterionic upon saturation of the DTPA binding sites, Bi-Am(EDA-DTPA)₄₀. This MCP was compared to Bi-Am(DTPA)₄₀ from the previously mentioned study and it concluded that zwitterionic MCPs represent a much better choice than polymers with charges along the backbone for in vivo delivery of RICs to HER2+ cancer cells [132].

Preserved immunoreactivity of mAbs linked to MCPs was demonstrated in the literature in a study describing the synthesis of MCPs and their linkage to trastuzumab where the immunoreactivity of the mAb for HER2 was preserved upon the linkage of the MCP as was shown with Kᵋ value of 6.9 ± 0.5 nM reported for trastuzumab linked to the MCP compared to 3.2 ± 1.2 nM for DTPA-modified trastuzumab [133]. The potential for increasing the specific activity of an antibody by linkage to MCPs was demonstrated in a subsequent study comparing trastuzumab modified with 2 DTPA chelation sites to trastuzumab modified with MCPs harbouring 24-29 DTPA chelation sites. In this study the maximum specific activities achieved were 0.1 MBq/µg (1.2 X 10⁴ MBq/µmol) and 8.9 MBq/µg (1.5 X 10⁶ MBq/µmol) [129].

Aghevlian et al. studied an MCP with a polyglutamide backbone harbouring 13 DOTA chelators for complexing to ¹¹¹In or ¹⁷⁷Lu with 10 PEG chains to minimize liver and spleen uptake conjugated to the intact IgG of panitumumab in pancreatic cancer xenograft mouse models and demonstrated the potential of MCP-RICs [56]. In this study, the bioconjugation of the MCP to panitumumab was achieved by reaction of a hydrazine nicotinamide (HyNIC) group on the MCP with an aryl aromatic aldehyde group introduced into panitumumab by reaction with succinimidyl-4-formylbenzamide (S-4FB). With this method of conjugation the reaction could be monitored by measurement of the chromophoric bis-aryl hydrozone bond formation (ε₃₅₀nm = 24 500 M⁻¹ cm⁻¹) and two MCPs per mAb was achieved. This MCP conjugated to panitumumab was compared to panitumumab derivatized with 2 DOTA groups. The study showed that the maximum specific activity was higher for MCP-panitumumab than for panitumumab labeled with either ¹¹¹In or ¹⁷⁷Lu, and that the immunoreactivity was preserved in that the Kᵋ for binding of panitumumab-MCP-¹⁷⁷Lu to EGFR (2.2 ± 0.6 nmol/L) was not significantly different than panitumumab-DOTA-¹⁷⁷Lu (1.0 ± 0.4 nmol/L). Also, there was comparable tumour accumulation for panitumumab-MCP-¹⁷⁷Lu and panitumumab-DOTA-¹⁷⁷Lu at 6.9 ± 1.3%ID/g and 6.6 ± 3.3%ID/g, respectively. Finally, this study showed that normal tissue distribution was
similar for except for panitumumab-MCP-^{177}\text{Lu} and panitumumab-DOTA-^{177}\text{Lu}, except for the liver accumulation which was 3-fold higher for the polymer RICs [56].

1.3 Nuclear medicine imaging of pancreatic cancer

Nuclear imaging modalities can be used for improved early detection of pancreatic cancer and monitoring of therapeutic response. Nuclear imaging has the unique ability to assess the phenotypic signature of a tumour non-invasively, and is also able to visualize biological processes such as tumour metabolism, angiogenesis, hypoxia, proliferation, as well as receptor expression. These imaging modalities include positron emission tomography (PET) and single-photon emission computed tomography (SPECT). Incorporation of CT with SPECT or PET imaging modalities enhances disease detection by accounting for attenuation, resolution effects, and motion artifacts [134, 135]. Several studies have demonstrated improvements in disease detection and treatment monitoring with combined imaging modalities, as compared to single imaging techniques [136-138].

PET imaging is a tomographic nuclear medicine modality that generates 3D data sets of the \textit{in vivo} distribution of radiopharmaceuticals labeled with positron emitting radioisotopes. The annihilation of a positron occurs upon collision with an electron and leads to two coincident gamma photons with energy of 511 keV that are then simultaneously emitted in opposite directions (angle of approximately 180 degrees). PET cameras are typically composed of a 360-degree ring of inorganic scintillation crystal detectors and subsequent photomultiplier tubes [139]. Two scintillation detectors separated by 180 degrees will transmit a coincident signal when they detect a 511 keV \(\gamma\)-photon simultaneously [140]. The detectors absorb the photon energy which is re-emitted as visible light and detected by the photomultiplier tubes. The light signal detected by the photomultiplier tubes gets converted into an electrical current proportional to the incident photon energy. The registered events are reconstructed into a 3D image representing the spatial distribution of the radioactive source in the studied subject. PET imaging provides high sensitivity and excellent tissue penetration, which allows for quantitative detection of PET tracers in the picomolar range [141]. Several positron-emitting radionuclides have been evaluated as potential for imaging pancreatic malignancies, including \(^{11}\text{C},\ ^{18}\text{F},\ ^{64}\text{Cu},\ \text{and}\ ^{89}\text{Zr}\) [142-144].
PET combined with CT can increase the sensitivity for detection of metastatic disease in pancreatic cancer patients by 87% [145]. Figure 1.10 shows PET/CT detection of an occult liver metastasis in a 66-year old male who had been diagnosed with pancreatic adenocarcinoma by endoscopic ultrasound. The PET imaging agent $^{18}$F-labeled 2-fluoro-2-deoxy-D-glucose (FDG), whose accumulation at a site indicates increased metabolic activity, was used in PET/CT imaging where the investigators were able to detect a lesion in the left lobe of the liver that was not detected by CT alone. A biopsy of the lesion identified it to be metastatic adenocarcinoma [145]. Another radiotracer than has been investigated for imaging pancreatic cancer is $^{18}$F-labeled fluoroazomycinarabinoside (FAZA) which is capable of imaging hypoxia in solid tumours [146]. $^{18}$F-FAZA is a second generation 2-nitroimidazole that has been shown to be hypoxia specific and reproducible [147]. Hypoxia is a pathological condition arising in living tissue when the oxygen supply does not adequately cover the cellular metabolic demand. This phenomenon is also present in the vast majority of solid tumours and has been associated with a tendency toward poor prognosis [148]. In a clinical study investigating $^{18}$F-FAZA as a radiotracer of hypoxia in pancreatic cancer patients the authors observe that although many pancreatic cancers were found to be hypoxic, a substantial proportion of tumours were well oxygenated. The demonstrated that $^{18}$F-FAZA would be useful in patient stratification in future clinical trials of hypoxia-targeting agents [149]. PET is useful in detecting metastatic disease, however, this imaging modality would not be helpful in determining the resectability of pancreatic cancer tumours due to the low spatial resolution of 5-10 mm.

While PET imaging relies upon the detection of positron-emitting isotopes, SPECT imaging detects single $\gamma$-radiation using an array of gamma cameras [150]. Several 2D projections of the patient are acquired at multiple angles and later reconstructed using tomographic reconstruction algorithms to form a 3D image of radiotracer biodistribution [151]. While PET imaging technologies provide superior resolution and quantitative abilities, SPECT technologies are more accessible in the clinic at a lower cost for patients [152]. Some common gamma emitters employed for SPECT imaging include $^{99m}$Tc, $^{111}$In, and $^{123}$I [153]. Currently, SPECT/CT is not commonly employed for detection of pancreatic malignancies in the clinic, yet improved imaging agents may promote its use in the future.
Figure 1.10. Transverse view of PET/CT detection of an occult liver metastasis with FDG as the radiotracer. A CT scan (A) of a patient diagnosed with pancreatic adenocarcinoma by ultrasound did not detect metastases. The fusion of PET/CT scans (B) detected an occult liver metastasis that was biopsied and confirmed to be metastatic adenocarcinoma. Reprinted from Farma JM [145].
Recently, clinical imaging of mesothelin-expressing pancreatic cancer was monitored in six patients using an $^{111}$In-labeled chimeric monoclonal antibody, amatuximab [154]. This study investigated $^{111}$In-amatuximab in four patients with malignant mesothelioma and two patients with pancreatic adenocarcinoma, and produced a tumour to background ratio $\geq$1.2, which was found to be sufficient in distinguishing between tumour and normal tissue. Furthermore, this was the first clinical trial examining the safety and biodistribution of $^{111}$In-amatuximab, and the imaging tracer displayed a favorable dosimetry profile and was tolerated well in patients.

### 1.4 Resistance of pancreatic cancer to treatment

Pancreatic cancer exhibits intrinsic resistance to chemotherapy with GEM, despite this being the first-line therapy [155]. Factors that contribute to the chemoresistance of pancreatic cancers are very diverse and many are unrelated, ranging from DNA repair, presence of resistant cancer stem cells, to the dense stroma and hypoxia found in the tumour microenvironment [156-158].

Pancreatic cancer patients often have a mutation in the tumour suppressor protein p53 gene (p53) [159]. The tumour suppressor protein p53 is a transcription factor that regulates the rate of genetic information relayed from DNA to messenger RNA. P53 can promote cell cycle arrest by regulating the G1/S checkpoint and initiating apoptosis [160]. The loss of function of this key cell cycle regulator can lead to uncontrollable cell division and tumour progression. Additionally, p53 mutation has been suggested to cause pancreatic cancer cells to become radioresistant [161]. GEM has been shown to stimulate the activity of the p53 mutant, and hence, the presence of a p53 mutation leads to resistance to GEM and hyperproliferation of pancreatic cancer cells [162]. The precise mechanism of GEM stimulating p53 mutant activity remains unresolved, but recently, one study demonstrated the restoration of p53 wild-type activity improves the response of pancreatic cancer cells to GEM [162].

In addition to chemoresistance, pancreatic cancer tumours are also resistant to treatment with radiation [163]. Indeed, pancreatic cancer cell lines have been demonstrated to have resistance to radiation to varying degrees, some with very high levels of resistance [155, 164]. Porcelli et al. demonstrated the magnitude and variability of pancreatic cancer cell response to $\gamma$-irradiation ($\gamma$-IR), finding that PANC-1 cells are more radioresistant to $\gamma$-IR than MiaPaCA-2, Capan-1, and AsPC-1 pancreatic cancer cells. In these studies, *in vitro* cell proliferation of PANC-1 could not
be reduced below 70% with doses as high as 10 Gy, while the proliferation of MiaPaCA-2, Capan-1, and AsPC-1 were reduced to 10-30% [164].

Although pancreatic cancer exhibits intrinsic resistance to chemotherapy with GEM and γ-IR [155] GEM has been demonstrated to act as a radiosensitizer for X- and γ-IR, as well as for RIT with an $^{131}$I-labeled anti-MUC1 mAb against pancreatic cancer tumours [105, 165, 166]. Indeed, a phase I clinical trial demonstrated improved survival for patients with locally advanced pancreatic cancer receiving the combination of GEM plus external beam radiotherapy compared to patients receiving GEM alone [167]. A meta-analysis of 15 randomized clinical trials investigating combination external radiation and chemotherapy (CRT) recently reported that CRT is more effective than either radiation or chemotherapy alone in the treatment of locally advanced pancreatic cancer [168]. More recently, many preclinical studies have combined GEM with another radiosensitizer and/or a GEM-sensitizer, for treatment of pancreatic cancer tumours in mice with γ-IR or RIT [101, 166, 169, 170]. For example, GEM and its metabolites activate checkpoint kinase 1 (CHK1) [101]. CHK1 coordinates DNA damage response and cell cycle checkpoint response [171]. The majority of pancreatic cancer cells are reliant on the S and G2 checkpoints for DNA repair, due to p53 mutation which interferes with G1 phase cell cycle arrest, a state which can be abrogated by CHK1 inhibitors (CHK1i) [101]. Al-Ejeh et al. demonstrated that the combination of RIT with $^{177}$Lu-labeled anti-EGFR mAb and chemotherapy with GEM plus a CHK1i, PF-477736, caused tumour growth arrest in mice engrafted with PANC-1 pancreatic cancer xenografts [101]. Another combination that has shown improved radiosensitizing effects is metformin (MET) combined with GEM and γ-IR. In these studies the combination of MET + GEM + γ-IR reduced the clonogenic survival of MiaPaca-2 pancreatic cancer cells 4-fold more effectively than γ-IR alone while GEM + γ-IR, MET + γ-IR were found to be 2-fold more effective than γ-IR [166]. This study also showed that the radiosensitizing ability of this combination was not observed when the adenosine 5′-monophosphate-activated kinase (AMPK) pathway was inhibited by compound C, indicating that AMPK signaling is involved in the radiosensitizing action of MET [166]. These results suggest that MET radiosensitizes pancreatic cancer cells through effecting metabolic regulation, a major role of AMPK signaling [166]. A third combination that has been demonstrated to have potent radiosensitizing effects preclinically both in vitro and in vivo involves an inhibitor of DNA signaling, an ataxia-telangiectasia and Rad3-related inhibitor (ATRi), with GEM and γ-IR [169,
ATR is known to activate CHK1, and so an ATRi would inhibit DNA repair during S and G2 checkpoints [169]. A fourth combination that has shown potent radiosensitizing effects preclinically in vitro is GEM the poly ADP ribose polymerase inhibitor (PARPi), rucaparib [164]. PARP1 is a protein that repairs DNA single-strand breaks (SSBs), so the addition of a PARPi to primary therapy may lead to DNA DSBs upon DNA replication [164].

A phase II clinical trial has been completed, with results not yet published, investigating the combination of GEM, anti-EGFR cetuximab, 5-FU, and external radiation therapy (ClinicalTrials.gov Identifier: NCT00424827). A phase II clinical trial is currently recruiting pancreatic cancer patients harboring BRCA1, BRCA2, PALB2, CHEK2, or ATM mutations to investigate a PARPi, niraparib, as an adjuvant therapy following primary treatment with chemotherapy and/or surgical resection of the tumour (ClinicalTrials.gov Identifier: NCT03601923). This PARPi could readily be studied as an adjuvant therapy following radiation therapy.

1.5 Hypothesis

Theranostic agents targeted to EGFR overexpression in pancreatic cancer cells would be useful for imaging, and for treating pancreatic cancer cells or tumours when combined with radiosensitizing chemotherapeutic drugs.

1.6 Specific aims

To test the hypothesis of this thesis the specific aims were:

1) To develop and characterize $^{64}$Cu-labeled panitumumab in its Fab, F(ab')$_2$, and intact IgG forms to assess tumour and normal tissue biodistribution, and perform microPET/CT imaging to demonstrate diagnostic potential in a clinically relevant patient-derived orthotopic pancreatic cancer xenograft model, as described in Chapter 2.

2) To develop three MCPs with 17 polyglutamamide (PGlu) repeat units, 30 DOTA chelators and with an average of 2, 4, and 8 pendant PEG$_{2K}$ chains and conjugate these MCPs to panitumumab F(ab')$_2$ fragments for radiolabeling with $^{64}$Cu, and to examine the in vitro and in vivo characteristics of the RICs and their potential for microPET/CT imaging, as described in Chapter 3.
3) To investigate the normal tissue toxicity and therapeutic efficacy of $^{64}$Cu-panitumumab F(ab′)$_2$ fragments combined with radiosensitizing chemotherapeutic drugs in vivo to study the therapeutic potential of $^{64}$Cu as a PET theranostic agent, as described in Chapter 4.

4) To examine $^{64}$Cu- and $^{177}$Lu-panitumumab F(ab′)$_2$ fragments in vitro compared to external radiation therapy (XRT) with γ-irradiation (γ-IR) in 4 pancreatic cancer cell lines with varying degrees of radiosensitivity and EGFR expression, to determine the RBE correlation to EGFR density, cell doubling time, and nuclear localization as described in Chapter 5.
Chapter 2
MicroPET/CT Imaging of Patient-Derived Pancreatic Cancer Xenografts Implanted Subcutaneously or Orthotopically in NOD-scid Mice using $^{64}\text{Cu}$-NOTA-Panitumumab F(ab’)$_2$ Fragments

All experiments and analyses of data were carried out by Amanda Jean Boyle. Patient-derived orthotopic pancreatic mouse models were prepared by Ping-Jiang Cao.
MicroPET/CT Imaging of Patient-Derived Pancreatic Cancer Xenografts Implanted Subcutaneously or Orthotopically in NOD-scid Mice using $^{64}$Cu-NOTA-Panitumumab F(ab’)$_2$ Fragments

2.1 Abstract

Introduction: Our objective was to study microPET/CT imaging of patient-derived pancreatic cancer xenografts in NOD-scid mice using F(ab’)$_2$ fragments of the fully-human anti-EGFR monoclonal antibody, panitumumab (Vectibix) labeled with $^{64}$Cu. More than 90% of pancreatic cancers are EGFR-positive.

Methods: F(ab’)$_2$ fragments were produced by proteolytic digestion of panitumumab IgG or non-specific human IgG, purified by ultrafiltration then modified with NOTA chelators for complexing $^{64}$Cu. Panitumumab IgG and Fab fragments were similarly labeled with $^{64}$Cu. EGFR immunoreactivity was determined in competition and direct (saturation) cell binding assays. The biodistribution of $^{64}$Cu-labeled panitumumab IgG, F(ab’)$_2$ and Fab were compared in non-tumour-bearing Balb/c mice. MicroPET/CT and biodistribution studies were performed in NOD-scid mice engrafted subcutaneously (s.c.) or orthotopically with patient-derived OCIP23 pancreatic tumours, or in athymic mice with s.c. PANC-1 human pancreatic cancer xenografts.

Results: Panitumumab F(ab’)$_2$ fragments were produced in high purity (>90%), derivatized with 3.2 ± 0.7 NOTA/ F(ab’)$_2$, and labeled with $^{64}$Cu (0.3-3.6 MBq/µg). The binding of $^{64}$Cu-NOTA-panitumumab F(ab’)$_2$ to OCIP23 or PANC-1 cells was decreased significantly by an excess of panitumumab IgG. The $K_d$ for binding of $^{64}$Cu-NOTA-panitumumab F(ab’)$_2$ to EGFR on PANC-1 cells was 0.14 ± 0.05 nmol/L. F(ab’)$_2$ fragments exhibited more suitable normal tissue distribution for tumour imaging with $^{64}$Cu than panitumumab IgG or Fab. Tumour uptake at 48 h p.i. of $^{64}$Cu-NOTA-panitumumab F(ab’)$_2$ was 12.0 ± 0.9% injected dose/g (ID/g) in s.c. and 11.8 ± 0.9% ID/g in orthotopich OCIP23 tumours vs. 6.1 ± 1.1% ID/g in s.c. PANC-1 xenografts. Tumour/blood (T/B) ratios were 5:1 to 9:1 for OCIP23 and 2.4:1 for PANC-1 tumours. Tumour uptake of $^{64}$Cu-NOTA-non-specific F(ab’)$_2$ in OCIP23 xenografts was 5-fold lower than $^{64}$Cu-panitumumab F(ab’)$_2$. All tumour xenografts were clearly imaged by microPET/CT at 24 or 48 h p.i. of $^{64}$Cu-NOTA-panitumumab F(ab’)$_2$. 
Conclusions: $^{64}$Cu-panitumumab F(ab′)2 fragments bound with high affinity to EGFR on pancreatic cancer cells in vitro and localized specifically in patient-derived pancreatic cancer xenografts in mice in vivo, allowing tumour visualization by microPET/CT at 24 or 48 h p.i.

Advances in Knowledge and Implications for Patient Care: $^{64}$Cu-panitumumab F(ab′)2 fragments imaged EGFR-positive patient-derived pancreatic cancer xenografts in mice by microPET/CT. The emission of positrons ($\beta^+$), $\beta$-particles ($\beta^-$), and Auger electrons by $^{64}$Cu may enable combined PET imaging and radioimmunotherapy (RIT) of pancreatic cancer using the same agent (“PET theranostics”).

2.2 Introduction

Adenocarcinoma of the pancreas (pancreatic cancer) is one of the most lethal cancers due to its often advanced stage at diagnosis and the limited treatment options available [172]. The 5-year survival from pancreatic cancer is only 5% [172]. Surgical resection offers the only opportunity for long-term survival, but less than 20% of patients are candidates for surgical treatment due to local vascular invasion or metastasis [173]. Only limited progress has been made in the management of pancreatic cancer, mainly through improvements in surgical management which have reduced the perioperative mortality rate to 5% [173] and the introduction of gemcitabine in an adjuvant setting which has increased the survival of surgically-treated patients [174]. There is a need for new diagnostic tools to improve the detection and staging of pancreatic cancer as well as more effective treatments for the disease in order to improve patient outcomes.

Our group is exploring a novel approach which will combine positron-emission tomography (PET) for detection of pancreatic cancer with radioimmunotherapy (RIT) using monoclonal antibodies (mAbs) labeled with $^{64}$Cu (“PET theranostics”). $^{64}$Cu is a versatile radionuclide since it emits positrons [E$\beta^+$ = 0.65 MeV (17.4%)] that enable PET as well as negatrons [E$\beta^-$ = 0.57 MeV (39.0%)] and low energy Auger electrons that allow RIT. Indeed, several investigators have shown the potential of $^{64}$Cu-labeled anti-tumour mAbs or peptides for targeted radiotherapy of malignancies [175-177]. In this report, we describe the development of $^{64}$Cu-labeled panitumumab F(ab′)2 fragments as potential PET theranostics. We demonstrate for the first time their ability to visualize by PET imaging patient-derived pancreatic cancer xenografts (PDX) implanted subcutaneously (s.c.) or orthotopically in non-obese diabetic severe combined
immunodeficiency (NOD-scid) mice. Panitumumab (Vectibix; Amgen) is a fully-human IgG2 mAb that binds the human epidermal growth factor receptor (EGFR) with high affinity [178, 179] and has been studied clinically for treatment of EGFR-positive malignancies [180]. EGFR is overexpressed on >90% of pancreatic cancers [181]. The efficient internalization reported for other anti-EGFR mAbs (e.g. cetuximab) may provide an opportunity to exploit the Auger electron emissions of $^{64}$Cu in addition to its $\beta^-$ emissions for RIT [182]. Furthermore, since panitumumab is a fully human mAb, this would permit repeated administration of $^{64}$Cu-labeled panitumumab F(ab')$_2$ fragments for PET or RIT without major concern for immunogenicity, which has previously been a limitation of murine or chimeric mAbs [183]. Moreover, PET offers high sensitivity and high spatial resolution and the images are quantifiable, which is attractive for determining tumour uptake in a future PET theranostic strategy [184].

Our objective in this study was to evaluate the specific uptake of $^{64}$Cu-labeled panitumumab F(ab')$_2$ fragments in pancreatic cancer PDX implanted s.c. or orthotopically in NOD-scid mice by microPET/CT imaging. These PDX models closely recapitulate the histologic features of the patient tumours from which they were derived, rendering them highly relevant preclinical models of human pancreatic cancer, particularly when implanted orthotopically into the pancreas. From the orthotopic site, tumours give rise to locoregional invasion and distant metastases including to the liver, lung and peritoneum (with ascites) [185]. Ultimately, our aim is to perform PET/CT imaging in pancreatic cancer patients with $^{64}$Cu-labeled panitumumab F(ab')$_2$ fragments in order to detect and stage the disease and to predict response to RIT with the same agents administered at a higher dose (“PET theranostics”).

2.3 Materials and methods

2.3.1 Cell culture and flow cytometry

PANC-1 human pancreatic adenocarcinoma cells (4.0 $\times$ 10$^5$ EGFR/cell) [186] and MDA-MB-468 human breast cancer cells (1.3 $\times$ 10$^6$ EGFR/cell) [187] were purchased from the American Type Culture Collection (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 streptomycin and 10% fetal bovine serum (FBS; Invitrogen, Burlington, ON, Canada). MDA-MB-468 cells were cultured in RPMI 1640 medium.
(Sigma-Aldrich, St. Louis, MO, USA) with 10% FBS and 1% penicillin/streptomycin
(Invitrogen). Patient-derived OCIP23 pancreatic tumours expressing EGFR were passaged in
NOD-scid mice as previously reported [188]. OCIP23 is a poorly differentiated pancreatic
adenocarcinoma with 100% K-ras mutation (codon 12) and p53-mutation and exhibits rapid
growth in NOD-scid mice [189]. For EGFR binding assays and flow cytometry, explanted
OCIP23 cells were briefly cultured in DMEM and Ham’s F-12 media supplemented with 7.5%
FBS. All cells were maintained in an atmosphere of 5% CO<sub>2</sub> at 37°C. The relative EGFR density
of OCIP23 compared to PANC-1 and MDA-MB-468 cells was assessed by flow cytometry.
Approximately 5 × 10<sup>5</sup> cells in 0.5 mL of PBS were incubated at 4°C with 1.25 μg of
panitumumab (Vectibix®; Amgen, Thousand Oaks, CA, USA) for 60 min, then with AlexaFluor
488 anti-human IgG immunoconjugates (Molecular Probes, Carlsbad, CA, USA) for 30 min at
4°C. Mean fluorescence intensity was then determined by flow cytometry collecting 15,000
events per cell line.

2.3.2 Antibody fragments
Panitumumab Fab fragments (MW ≈ 55 kDa) were prepared as previously reported for
trastuzumab Fab fragments [190] by proteolysis of 5 mg of panitumumab IgG (MW ≈ 147 kDa;
purchased from the Pharmacy Department, University Health Network, Toronto, ON, Canada)
with 10 mL immobilized-papain slurry (Pierce, Rockford, IL, USA). Panitumumab F(ab’)<sub>2</sub>
fragments (M<sub>r</sub> ≈ 110 kDa) were prepared by proteolysis of IgG with immobilized pepsin
(Pierce). Immobilized pepsin was equilibrated in 20 mM sodium acetate trihydrate buffer (pH
4.5) then combined with panitumumab IgG at 4 mg per 0.25 mL slurry, and finally incubated for
5 h at 37°C on a Nutating Mixer (VWR, Mississauga, ON, Canada). The mixture was
centrifuged at 1000 × g for 5 min, the supernatant was collected and passed through a Millex®-
GV PDVF 0.22 μm filter. The resin was rinsed twice by resuspending in 1.0 mL PBS (pH 7.4),
centrifuged again, then collected and filtered and pooled as described above. Panitumumab
F(ab’)<sub>2</sub> and Fab fragments were re-concentrated to 5 mg/mL in PBS (pH 7.4) on an Amicon Ultra
device (Millipore; M<sub>r</sub> cut-off = 30 kDa). The purity of panitumumab Fab and F(ab’)<sub>2</sub> fragments
was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on a
7.5% Tris-HCl gel (Bio-Rad) under reducing and non-reducing conditions stained with Bio-Safe
Coomassie G-250 stain (Bio-Rad). In addition, size-exclusion high performance liquid
chromatography (SE-HPLC) was performed on a BioSep SEC-S2000 column (Phenomenex, Torrance, USA) eluted with 0.1 M NaH₂PO₄ buffer (pH 7.0) at a flow rate of 1.0 mL/min using a Waters 2695 HPLC system interfaced with a diode array detector set at 280 nm (Waters Inc., Mississauga, ON). F(ab’)₂ fragments of non-specific human IgG (hIgG) from human serum (Product I4506, Sigma-Aldrich) were similarly prepared and analysed for purity.

2.3.3 NOTA conjugation and labeling with ⁶⁴Cu

NOTA was conjugated to panitumumab IgG, Fab or F(ab’)₂ fragments by reaction with p-SCN-Bn-NOTA (Macrocyclics, Dallas, TX, USA). p-SCN-Bn-NOTA was dissolved in 5 μL dimethyl sulfoxide (DMSO) then diluted to a final concentration of 1 mg/mL in 100 mM NaHCO₃ (pH 9.2) and sonicated for 30 sec. Intact panitumumab, Fab or F(ab’)₂ fragments were buffer-exchanged and concentrated to 5 mg/mL in 100 mM NaHCO₃ (pH 9.2) using an Amicon Ultra device (Millipore; Mₜ cut-off = 10 kDa). All buffers used for NOTA conjugation or ⁶⁴Cu labeling were pre-treated with Chelex-100 resin (Bio-Rad, Mississauga, ON, Canada) to remove cationic trace metals as previously reported [191]. Buffer-exchanged intact antibodies or Fab or F(ab’)₂ fragments (5 mg/mL) were then reacted with a 10-fold molar excess of p-SCN-Bn-NOTA for 2 h at room temperature (RT) then incubated overnight at 4°C. The p-SCN-Bn-NOTA-derivated immunoconjugates were purified from excess p-SCN-Bn-NOTA using an Amicon Ultra device (Millipore; Mₜ cut-off = 30 kDa). The NOTA-substitution level of the immunoconjugates (mols NOTA/mol IgG, Fab or F(ab’)₂ fragment) was determined by the copper(II)-arsenazo(III) assay with minor modifications [192]. ⁶⁴Cu-labeling was achieved by incubating 10 μg of immunoconjugates (5 mg/mL) with 4-37 MBq of ⁶⁴CuCl₂ (Washington University, St. Louis, MO, USA) for 1 h at 40°C. The final radiochemical purity was measured by instant thin-layer silica gel chromatography (ITLC-SG; Pall Life Sciences, Ann Arbor, MI, USA), developed in 100 mM sodium citrate (pH 5.0). The Rᵣ value of ⁶⁴Cu-labeled IgG, Fab or F(ab’)₂ fragments in this system is 0.0 and that of free ⁶⁴Cu or ⁶⁴Cu-NOTA is 1.0. In addition, SE-HPLC was performed on a BioSep SEC-S2000 column (Phenomenex) eluted with 0.1 M NaH₂PO₄ buffer (pH 7.0) at a flow rate of 1.0 mL/min using a Waters 2695 HPLC system interfaced with a diode array detector set at 280 nm and a Flow-Count radioactivity detector (BioScan Inc., Washington, DC, USA). F(ab’)₂ fragments of non-specific hIgG were similarly derivatized with NOTA and labeled with ⁶⁴Cu and analyzed for radiochemical purity.
2.3.4 EGFR binding of radioimmunoconjugates

The binding of $^{64}$Cu-labeled panitumumab and non-specific F(ab')$_2$ fragments to EGFR-positive PANC-1 or OCIP23 pancreatic cancer cells was measured in single concentration (“one-point”) competition binding assays. Approximately $1.5 \times 10^5$ cells were seeded into wells in a 24-well, flat bottom, tissue culture plate (Sarstedt, Montreal, QC, Canada) and cultured overnight. The growth medium was removed and the cells were incubated with 2.5, 5 or 10 nmoles/L of the radioimmunoconjugates (1-3 MBq/μg) contained in 0.5 mL of serum-free medium for 3 h at 4°C. Non-specific binding (NSB) was estimated by repeating the assay in the presence of a 100-fold molar excess of panitumumab IgG. The medium was removed and the adherent cells were rinsed with PBS (pH 7.4) then solubilized in 100 mM NaOH at 37°C for 30 min. The dissolved cells were transferred to γ-counting tubes and the total cell-bound radioactivity (TB) was measured in a γ-counter (Model 1480; PerkinElmer, Massachusetts, USA). Specific binding (SB) was calculated by subtracting NSB from TB. EGFR binding (NSB and SB) was expressed as the percent of TB. A direct saturation binding assay using PANC-1 cells was performed to measure the dissociation constant ($K_d$) and maximum number of binding sites per cell ($B_{max}$) for $^{64}$Cu-labeled panitumumab F(ab')$_2$ fragments. For this assay, increasing concentrations of $^{64}$Cu-labeled panitumumab F(ab')$_2$ fragments (1.2-300 nmol/L) were incubated with $1.5 \times 10^5$ cells in wells in a 24-well polystyrene culture plate for 3 h at 4°C. These assays were repeated with a 50-fold molar excess of panitumumab IgG to estimate NSB. The medium removed from the adherent cells was collected and transferred to γ-counting tubes and the amount of cell-bound radioactivity was measured in a γ-counter. SB was calculated by subtracting NSB from TB. The data was plotted as the amount of $^{64}$Cu-panitumumab F(ab')$_2$ fragments bound (pmol) vs. the concentration (nmol/L) of free $^{64}$Cu-panitumumab F(ab')$_2$ fragments. The binding curve was fitted to a one-site receptor-binding model using Prism Ver. 4.0 software (GraphPad, San Diego, CA, USA). This assay was repeated three times and $K_d$ and $B_{max}$ values were reported as the mean ± SEM.

2.3.5 Biodistribution studies

The normal tissue biodistribution of $^{64}$Cu-labeled panitumumab IgG, Fab, and F(ab')$_2$, fragments were compared in non-tumour-bearing Balb/c mice in order to identify the most suitable form for microPET/CT imaging. Groups of 3-4 mice were injected i.v. (tail vein) with 4 MBq (10 μg) of
radioimmunoconjugates, then sacrificed by cervical dislocation under anaesthetic [ketamine/xylazine/acepromazine (100 mg/kg; 5 mg/kg; 1 mg/kg)] at 18 h post-injection (p.i.). Blood and selected tissue samples were collected, weighed, and transferred to γ-counting tubes. Tissue radioactivity was measured in a γ-counter and expressed as the percent injected dose per gram (% ID/g). Based on the results of these studies, $^{64}$Cu-labeled panitumumab F(ab')$_2$ fragments were selected for biodistribution studies in tumour-bearing mice. Tumour xenograft mouse models were prepared using PANC-1 cells, and patient-derived OCIP23 tumours. NOD-scid mice were inoculated subcutaneously (s.c.) with $1 \times 10^7$ PANC-1 cells in 100 μL serum-free media on the right flank. OCIP23 tumours were passaged in NOD-scid mice that were inoculated s.c. on the right flank or at the orthotopic pancreas site with tumour fragments via a small incision in the upper left abdomen, as previously reported [188]. Tumour-bearing mice were sacrificed at 48 h p.i. of $^{64}$Cu-labeled panitumumab or non-specific F(ab')$_2$ fragments. The tumour and samples of blood and normal tissues were collected, weighed and their radioactivity measured in a γ-counter and expressed as % ID/g. All animal studies were conducted under a protocol (#2843.0) approved by the Animal Use Committee at the University Health Network following Canadian Council on Animal Care (CCAC) guidelines.

### 2.3.6 MicroPET/CT imaging studies

MicroPET was performed at 24 and 48 h post i.v. (tail vein) injection of 20-37 MBq (10 μg) of $^{64}$Cu-labeled panitumumab F(ab')$_2$ fragments in groups of 3-5 tumour-bearing mice on a Focus 220 microPET tomograph (Siemens Preclinical Solutions, Knoxville, TN, USA). Anaesthesia was induced and maintained by 2% isoflurane in O$_2$. Images were acquired for 15-90 min and reconstructed using OSEM, followed by a maximum a posteriori probability reconstruction algorithm with no correction for attenuation or partial-volume effects. The full width at half maximum (FWHM) resolution of the microPET tomograph was 1.6 mm. Immediately after imaging, CT was performed on an eXplore Locus Ultra Preclinical CT scanner (GE Healthcare, Mississauga, ON, Canada) with routine acquisition parameters (80 kVp, 70 mA, and voxel size of $150 \times 150 \times 150$ mm). MicroPET and CT images were co-registered using Inveon Research Workplace software (Siemens). The EGFR expression of orthotopic OCIP23 tumour xenografts was assessed by immunohistochemical staining using anti-human EGFR antibodies (Zymed Cat. No. 28-8763; Invitrogen Life Technologies, Carlsbad, CA, USA) as previously reported [193].
2.3.7 Statistical analysis

Data are represented as the mean ± SEM. Statistical comparisons were performed using an unpaired t-test (p < 0.05) using Prism Version 4.0 software (GraphPad).

2.4 Results

2.4.1 $^{64}$Cu-labeled panitumumab F(ab’)$_2$ fragment preparation

F(ab’)$_2$ fragments were obtained in high purity (>90%) by proteolytic digestion of panitumumab IgG using immobilized pepsin, assessed by SE-HPLC analysis (Figure 2.1A). SDS-PAGE (Figure 2.1B) demonstrated that the F(ab’)$_2$ fragments migrated with the expected molecular size under non-reducing conditions (apparent MW ≈ 110 kDa) or reducing conditions (apparent MW ≈ 20 kDa). Fab fragments similarly exhibited high purity (>90%) by SE-HPLC and SDS-PAGE analyses (not shown). Reaction of 5 mg/mL panitumumab Fab, F(ab’)$_2$, or IgG with p-SCN-Bn-NOTA resulted in substitution of 1.4 ± 0.1 NOTA/Fab (n=3), 3.2 ± 0.7 NOTA/F(ab’)$_2$ (n=3), and 4.2 ± 0.2 NOTA/IgG (n=3). The immunoconjugates were purified by ultrafiltration and labeled with $^{64}$Cu to a specific activity of 0.3–3.6 MBq/µg (33–396 MBq/mmole). No post-labeling purification was required and the final radiochemical purity of $^{64}$Cu-NOTA-panitumumab IgG, Fab or F(ab’)$_2$ was >95% as determined by SE-HPLC (Figure 2.1A) or ITLC-SG (not shown).

2.4.2 EGFR density of OCIP23 cells assessed by flow cytometry

The EGFR density of OCIP23 cells was estimated by flow cytometry by comparison to the EGFR density reported for PANC-1 (4.0 × 10$^5$ EGFR/cell) [186] or MDA-MB-468 cells (1.3 × 10$^6$ EGFR/cell) [187] (Figure 2.2). Based on the mean fluorescence intensity, it was estimated that there were approximately 4.3 × 10$^5$ EGFR/cell on OCIP23 cells.

2.4.3 EGFR binding of $^{64}$Cu-labeled panitumumab F(ab’)$_2$

In single concentration ("one-point") competition binding assays, the binding of $^{64}$Cu-NOTA-panitumumab F(ab’)$_2$ (10 nmol/L) to PANC-1 cells was reduced to 5.3 ± 0.2% in the presence of 100-fold molar excess of panitumumab IgG (Figure 2.3A). The binding of $^{64}$Cu-labeled panitumumab F(ab’)$_2$ to OCIP23 cells (4.3 × 10$^5$ EGFR/cell) at three different concentrations
Figure 2.1. HPCL and SDS-PAGE analysis of $^{64}$Cu-panitumumab F(ab′)$_2$ fragments. A) SE-HPLC analysis of $^{64}$Cu-labeled panitumumab F(ab′)$_2$ fragments detected by a diode array detector set at 280 nm (blue line) and radioactivity detector (black line). The retention time ($t_R$) of $^{64}$Cu-NOTA-panitumumab F(ab′)$_2$ by UV detection was 17.8 min. Using radioactivity detection, the $t_R$ $^{64}$Cu-NOTA-panitumumab F(ab′)$_2$ was 19.6 min. There is a delay between the diode array and radioactivity detectors which accounts for the different retention times. The small peak at $t_R$=20.5 mins (UV) or $t_R$=21.0 mins (radioactivity) represents free $^{64}$Cu-NOTA (<5%). The larger flow cell of the radioactivity detector causes peak broadening. B) SDS-PAGE analysis of panitumumab F(ab′)$_2$ fragments under non-reducing (lane 1) and reducing (lane 2) conditions. A protein ladder for standard molecular weights (indicated) is also shown (MW).
Figure 2.2. EGFR-density of cancer cells analyzed by flow cytometry following staining with primary panitumumab human IgG\textsubscript{2} monoclonal antibodies followed by AlexaFluor 488 goat anti-human IgG secondary antibodies (red line) or AlexaFluor 488 goat anti-human IgG secondary antibodies alone (blue line). A) OCIP23 patient-derived pancreatic cancer cells. B) PANC-1 human pancreatic cancer cells (4.0 \times 10^5 EGFR/cell). C) MDA-MB-468 human breast cancer cells (1.3 \times 10^6 EGFR/cell).
Figure 2.3. Binding of $^{64}$Cu-labeled panitumumab F(ab')$_2$ fragments or non-specific F(ab')$_2$ fragments to human pancreatic cancer cells in the absence (total binding; TB) or presence (non-specific binding; NSB) of a 100-fold molar excess of unlabeled panitumumab IgG. Specific binding (SB) was calculated by subtracting NSB from TB. Binding was expressed as the mean ± SEM (n=3) of the percent of TB (set to 100%). A) PANC-1 cells incubated with 10 nmol/L of radioimmunoconjugates. Significant differences ($p<0.05$) are noted by asterisks. B) OCIP23 incubated with 10, 5 or 2.5 nmol/L of radioimmunoconjugates.
(10 nmol/L, 5 nmol/L, or 2.5 nmol/L) was reduced to 35.4 ± 0.7%, 20.1 ± 2.0%, and 19.7 ± 0.3%, respectively in the presence of a 100-fold excess of panitumumab IgG (Figure 2.3B). A direct (saturation) binding assay was performed to estimate the dissociation constant ($K_d$) and the maximum number of binding sites ($B_{\text{max}}$) for binding of $^{64}\text{Cu}$-labeled panitumumab F(ab’)$_2$ to EGFR on PANC-1 cells (Figure 2.4). This assay demonstrated saturable binding to PANC-1 cells that was displaced by a 50-fold excess of panitumumab IgG. Fitting of the SB curve to a one-site receptor binding model for 3 independent assays revealed a mean $K_d = 0.14 ± 0.05$ nmol/L and $B_{\text{max}} = 3.7 \times 10^4 ± 1.6 \times 10^4$ receptors/cell.

### 2.4.4 Biodistribution studies

The normal tissue biodistribution at 18 h p.i. of $^{64}\text{Cu}$-NOTA-panitumumab Fab or F(ab’)$_2$ fragments or IgG were compared in non-tumour-bearing Balb/c mice (Figure 2.5). The kidney uptake of $^{64}\text{Cu}$-NOTA-panitumumab Fab fragments (90.8 ± 9.0% ID/g) was significantly greater ($p<0.005$) than for $^{64}\text{Cu}$-NOTA-panitumumab F(ab’)$_2$ (6.9 ± 0.9% ID/g) or $^{64}\text{Cu}$-NOTA-panitumumab IgG (11.5 ± 1.0% ID/g). The blood concentration of radioactivity for $^{64}\text{Cu}$-NOTA-panitumumab Fab (0.6 ± 0.1% ID/g) was 30-40 fold lower than for $^{64}\text{Cu}$-NOTA-panitumumab F(ab’)$_2$ (23.8 ± 10.5% ID/g; $p<0.05$) or $^{64}\text{Cu}$-NOTA-panitumumab IgG (18.2 ± 7.8% ID/g; $p<0.05$). The liver uptake of $^{64}\text{Cu}$-NOTA-panitumumab IgG (8.6 ± 0.9% ID/g) was 3-fold and 1.5-fold significantly greater than for $^{64}\text{Cu}$-NOTA-panitumumab Fab or F(ab’)$_2$ (2.5 ± 0.1% ID/g and 5.4 ± 0.2% ID/g, respectively; $p<0.05$). Low liver uptake, rapid elimination from the blood and low kidney uptake were desired for tumour imaging with $^{64}\text{Cu}$, therefore $^{64}\text{Cu}$-NOTA-panitumumab F(ab’)$_2$ fragments were selected for subsequent biodistribution and microPET/CT studies in tumour-bearing mice.

In NOD-scid mice bearing s.c. PANC-1 pancreatic cancer xenografts ($4 \times 10^5$ EGFR/cell), tumour uptake of $^{64}\text{Cu}$-NOTA-panitumumab F(ab’)$_2$ fragments was 7.0 ± 0.4% ID/g at 24 h p.i. and 6.2 ± 1.1% ID/g at 48 h p.i. ($p>0.05$; Table 2.1). Blood radioactivity significantly decreased from 6.0 ± 0.6% ID/g at 24 h p.i. to 2.6 ± 0.2% ID/g ($p<0.05$) at 48 h p.i. yielding a T/B ratio of 1:1 at 24 h p.i. and 2.4:1 at 48 h p.i. In NOD-scid mice implanted with OCIP23 PDX ($4.3 \times 10^5$ EGFR/cell) and injected with $^{64}\text{Cu}$-NOTA-panitumumab F(ab’)$_2$ fragments, uptake in s.c. tumours was 12.0 ± 0.9% ID/g and 11.9 ± 3.3% ID/g in tumours implanted orthotopically into the pancreas (Table 2.2). The uptake in locoregional metastases in the peritoneum (secondary
**Figure 2.4.** Direct (saturation) binding of $^{64}$Cu-NOTA-panitumumab F(ab')$_2$ fragments to EGFR on PANC-1 cells in the absence (total binding; TB) or presence (non-specific binding; NSB) of 50-fold molar excess of unlabeled panitumumab IgG. Specific binding (SB) was calculated by subtracting NSB from TB. Curves were fitted to a one-site receptor binding model using Prism Ver. 4.0 software (GraphPad). Error bars represent the mean ± SEM (n=3). In this representative assay, $^{64}$Cu-panitumumab F(ab')$_2$ fragments exhibited a $K_d=0.15$ nmol/L and $B_{max}=5.4 \times 10^4$ EGFR/cell.
Figure 2.5. Normal tissue distribution in non-tumour-bearing Balb/c mice at 18 h p.i. of $^{64}$Cu-labeled panitumumab Fab or F(ab$'$)$_2$ fragments or intact IgG. Values shown are the mean % injected dose per gram (%ID/g) ± SEM (n=3-5). Significant differences ($p<0.05$) in the concentrations of radioactivity in the blood, liver and kidneys are indicated by asterisks.
Table 2.1. Tumour and normal tissue biodistribution of $^{64}$Cu-NOTA-panitumumab F(ab')$_2$ fragments at 24 or 48 h p.i. in mice bearing subcutaneous PANC-1 human pancreatic cancer xenografts.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>6.0 ± 0.57 $^b$</td>
<td>2.6 ± 0.17 $^b$</td>
</tr>
<tr>
<td>Heart</td>
<td>2.4 ± 0.12</td>
<td>1.7 ± 0.21</td>
</tr>
<tr>
<td>Lungs</td>
<td>3.4 ± 0.38</td>
<td>2.6 ± 0.24</td>
</tr>
<tr>
<td>Liver</td>
<td>6.9 ± 0.44</td>
<td>6.5 ± 0.68</td>
</tr>
<tr>
<td>Spleen</td>
<td>5.4 ± 0.28</td>
<td>4.0 ± 0.14</td>
</tr>
<tr>
<td>Pancreas</td>
<td>1.2 ± 0.07</td>
<td>1.0 ± 0.07</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.0 ± 0.23</td>
<td>1.0 ± 0.23</td>
</tr>
<tr>
<td>Intestines</td>
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<td>1.7 ± 0.23</td>
</tr>
<tr>
<td>Kidneys</td>
<td>5.6 ± 0.65</td>
<td>4.6 ± 0.49</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.7 ± 0.21</td>
<td>0.3 ± 0.02</td>
</tr>
<tr>
<td>Tumour</td>
<td>7.0 ± 0.36</td>
<td>6.1 ± 1.11</td>
</tr>
</tbody>
</table>

$^a$ Mean ± SEM (n = 5)  
$^b$ Significantly different ($p<0.05$)
pancreatic tumours) in mice with orthotopic OCIP23 xenografts was 5.6 ± 1.7% ID/g. Blood concentrations of radioactivity at 48 h p.i. of $^{64}$Cu-NOTA-panitumumab F(ab')$_2$ fragments were 2.4 ± 0.2% ID/g in mice with s.c. OCIP23 tumours, and 1.4 ± 0.2% ID/g in mice with orthotopic tumours. T/B ratios were 5:1 for mice with s.c. OCIP23 tumours and 9:1 for mice with orthotopic tumours (5:1 for secondary tumours). NOD-scid mice implanted with OCIP23 tumours and injected with $^{64}$Cu-labeled non-specific F(ab')$_2$ had significantly lower tumour uptake at 48 h p.i. than for $^{64}$Cu-NOTA-panitumumab F(ab')$_2$ (2.3 ± 0.3% ID/g and 2.2 ± 0.1% ID/g, in s.c. and orthotopic tumour xenografts, respectively; $p$<0.05; Table 2.2).

2.4.5 MicroPET/CT imaging studies

MicroPET/CT imaging visualized s.c. PANC-1 pancreatic tumour xenografts and OCIP23 PDX implanted s.c. or orthotopically in NOD-scid mice at either 24 or 48 h p.i. of $^{64}$Cu-NOTA-panitumumab F(ab')$_2$ fragments (Figure 2.6A-C). Also visualized on the images was the liver which decreased slightly at 48 h p.i. and the bladder, which was likely due to renal excretion of $^{64}$Cu-NOTA-panitumumab F(ab')$_2$ fragments. The orthotopic OCIP23 tumours were visualized as a diffuse subphrenic accumulation of radioactivity. Liver or locoregional metastases from the primary OCIP23 tumours in the pancreas were not visualized in mice which were selected for imaging, but uptake in secondary tumours was identified in other mice in biodistribution studies (Table 2.2). Orthotopic OCIP23 tumour xenografts exhibited strong immunohistochemical staining for EGFR (Figure 2.6D).

2.5 Discussion

MicroPET/CT imaging at 24 or 48 h p.i. of $^{64}$Cu-NOTA-panitumumab F(ab')$_2$ fragments clearly visualized OCIP23 pancreatic cancer PDX implanted s.c. or orthotopically in NOD-scid mice. Tumour uptake in mice with s.c. or orthotopic OCIP23 xenografts at 48 h p.i. (12% ID/g; Table 2.2) was EGFR-specific since $^{64}$Cu-labeled non-specific F(ab')$_2$ fragments exhibited 6-fold significantly lower tumour uptake (Table 2.2). Accumulation (>5% ID/g) of $^{64}$Cu-panitumumab F(ab')$_2$ was also measured in secondary tumours that arose from locoregional spread within the peritoneum of OCIP23 xenografts implanted orthotopically in the pancreas (Table 2.2). EGFR-positive tumour xenografts established using the human pancreatic cancer cell line, PANC-1,
implanted s.c. into mice were also imaged with $^{64}$Cu-NOTA-panitumumab F(ab')$_2$ fragments. However, PDX implanted orthotopically in the pancreas most closely recapitulate the properties of the tumours in humans including locoregional invasion and metastasis to distant organs [185]. Thus, these tumour xenograft mouse models are most useful for assessing the biology of pancreatic cancer in patients and investigating new diagnostics and treatments for the disease [185, 188, 189]. To our knowledge, this study represents the first report of imaging of EGFR-positive pancreatic cancer in these more clinically-relevant mouse PDX tumour models by microPET/CT imaging using $^{64}$Cu-panitumumab F(ab')$_2$.

Both PANC-1 and OCIP23 cells displayed moderate EGFR density assessed in vitro by flow cytometry ($4 \times 10^5$ EGFR/cell; Figure 2.2), but there was unexpectedly 2-fold greater uptake at 48 h p.i. of $^{64}$Cu-NOTA-panitumumab F(ab')$_2$ in OCIP23 xenografts than in PANC-1 tumours (12% ID/g vs. 6% ID/g; Table 2.1 and 2.2). T/B ratios were also 2-4 fold higher for OCIP23 than PANC-1 tumours (5:1-9:1 vs. 2.4:1). In addition, OCIP23 xenografts were more intensely visualized on images than PANC-1 tumours (Figure 2.6 A-C). Orthotopic OCIP23 tumour xenografts exhibited strong immunohistochemical staining for EGFR (Figure 2.6D). Cai et al. reported a direct correlation between EGFR expression on tumour xenografts in athymic mice and the uptake of $^{64}$Cu-labeled anti-EGFR cetuximab mAbs [194]. Thus, it is possible that OCIP23 cells may exhibit higher EGFR density when grown in vivo as tumours in mice than when cultured in vitro. This phenomenon has been previously reported for LS174T human colon cancer cells in which the expression of tumour-associated glycoprotein-72 (TAG-72) was increased more than 100-fold when these cells were grown as tumours in mice than when cultured in vitro [195]. Furthermore, Milenic et al. reported very high uptake of $^{111}$In-labeled cetuximab in LS174T tumours (up to 52% ID/g) despite only modest EGFR expression by these cells assessed in vitro by flow cytometry [196]. They similarly propose that high tumour uptake of $^{111}$In-labeled cetuximab in vivo may be explained by upregulated EGFR expression by LS174T cells grown as tumour xenografts in mice.

F(ab')$_2$ fragments were selected for tumour imaging with microPET/CT because they were more rapidly eliminated from the blood and exhibited lower liver uptake than panitumumab IgG in non-tumour-bearing Balb/c mice (Figure 2.5). Rapid blood clearance is required to minimize the background for PET within the useful lifetime of $^{64}$Cu ($t_{1/2} = 12.7$ h). Low liver uptake would be
Table 2.2. Tumour and normal tissue biodistribution of $^{64}$Cu-NOTA-panitumumab F(ab')$_2$ or $^{64}$Cu-NOTA-non-specific F(ab')$_2$ at 48 h p.i. in mice bearing subcutaneous or orthotopic patient-derived OCIP23 human pancreatic cancer xenografts.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$^{64}$Cu-NOTA-panitumumab F(ab')$_2$</th>
<th>$^{64}$Cu-NOTA-non-specific F(ab')$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Subcutaneous</td>
<td>Orthotopic</td>
</tr>
<tr>
<td>Blood</td>
<td>2.4 ± 0.18</td>
<td>1.2 ± 0.39</td>
</tr>
<tr>
<td>Heart</td>
<td>1.7 ± 0.15</td>
<td>1.1 ± 0.28</td>
</tr>
<tr>
<td>Lungs</td>
<td>2.7 ± 0.25</td>
<td>1.7 ± 0.51</td>
</tr>
<tr>
<td>Liver</td>
<td>5.9 ± 0.14</td>
<td>4.2 ± 1.09</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.6 ± 0.43</td>
<td>2.3 ± 0.59</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.7 ± 0.04</td>
<td>1.8 ± 0.64</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.9 ± 0.08</td>
<td>1.1 ± 0.43</td>
</tr>
<tr>
<td>Intestines</td>
<td>1.3 ± 0.06</td>
<td>1.3 ± 0.20</td>
</tr>
<tr>
<td>Kidneys</td>
<td>3.6 ± 0.10</td>
<td>3.0 ± 0.69</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.4 ± 0.05</td>
<td>0.4 ± 0.12</td>
</tr>
<tr>
<td>Primary Tumour</td>
<td>12.0 ± 0.91 $^b$</td>
<td>11.8 ± 3.31 $^b$</td>
</tr>
<tr>
<td>Secondary Tumour</td>
<td>n.d.</td>
<td>5.6 ± 1.66</td>
</tr>
</tbody>
</table>

$^a$ Mean ± SEM (n = 3-5)

$^b$ Significantly different compared to corresponding value for $^{64}$Cu-NOTA-non-specific F(ab')$_2$ fragments ($p$<0.05).
Figure 2.6. Posterior whole-body coronal microPET/CT images at 24 or 48 h p.i. of $^{64}$Cu-NOTA-panitumumab F(ab’)$_2$ fragments in CD-1 athymic mice bearing human pancreatic cancer xenografts. The location of the tumour xenografts is indicated by the red circle. Also visualized on the images are the liver (blue arrowhead) and bladder (white arrowhead). A. Subcutaneous (s.c.) PANC-1 xenografts. B. OCIP23 patient-derived pancreatic cancer xenografts implanted s.c. C. OCIP23 xenografts implanted orthotopically into the pancreas. D. Immunohistochemical staining of OCIP23 xenografts demonstrating strong positivity for EGFR.
important for imaging hepatic metastases in patients with pancreatic cancer. F(ab')₂ fragments also exhibited dramatically lower kidney uptake than Fab fragments (Figure 2.5) which is important to minimize the radiation absorbed dose to the kidneys for future RIT applications of ⁶⁴Cu-NOTA-panitumumab F(ab')₂ fragments. The kidney uptake of ⁶⁴Cu-NOTA-panitumumab Fab fragments at 18 h p.i. in non-tumour bearing mice (90.8 ± 9.0% ID/g) was higher than we previously found for ⁶⁴Cu-DOTA-trastuzumab Fab fragments in mice with subcutaneous human breast cancer xenografts at 24 h p.i. (57.0 ± 7.1% ID/g) [191]. The high renal uptake of Fab fragments labeled with radiometals is thought to be due to interactions between cationic amino acids in the Fab fragments (e.g. lysine) and anionic charges present on the cell membrane of renal tubular cells; these interactions are inhibited by administration of lysine solutions [197]. Radiolabeled F(ab')₂ recognizing other tumour-associated epitopes have been studied clinically for RIT of malignancies without toxicity to the kidneys [198-200]. It should be appreciated that panitumumab binds to human EGFR and thus, normal tissue uptake mediated by binding to the murine EGFR homologue was not studied in this mouse tumour model [179].

NOTA was selected as a chelator because it forms stable complexes with ⁶⁴Cu [201]. Modification of panitumumab F(ab')₂ fragments with 3.2 ± 0.7 NOTA chelators preserved specific binding to EGFR on PANC-1 cells in a direct (saturation) binding assay (Kₐ = 0.14 ± 0.05 nmol/L; Figure 2.4) and to PANC-1 and OCIP23 cells in a one-point competition assay (Figure 2.3). Unmodified panitumumab IgG binds the EGFR with a Kₐ=0.05 nmol/L [202]. Wong et al. [203] found that panitumumab F(ab')₂ fragments modified with 1.7 to 5.6 CHX-A''-DTPA chelators for complexing ¹¹¹In or ⁸⁶Y similarly exhibited preserved EGFR binding. They reported very high uptake (>21% ID/g) of ¹¹¹In-CHX-A''-DTPA-panitumumab F(ab')₂ fragments in s.c. EGFR-positive LS174T tumour xenografts in mice at 24 or 48 h p.i. [203]. Tumour uptake of ⁶⁴Cu-NOTA-panitumumab F(ab')₂ fragments in our study was 2-3 fold lower (6-12% ID/g) which may reflect lower EGFR expression on PANC-1 and OCIP23 xenografts compared to LS174T tumours.

2.6 Conclusion

We conclude that ⁶⁴Cu-panitumumab F(ab')₂ fragments exhibited preserved high affinity binding to EGFR in vitro and localized specifically in vivo in pancreatic cancer PDX xenografts in NOD-scid mice. MicroPET/CT imaging at 24 or 48 h p.i. of ⁶⁴Cu-panitumumab F(ab')₂ clearly
visualized both s.c. and orthotopically implanted human pancreatic cancer xenografts. These results are promising for the application of $^{64}$Cu-panitumumab F(ab$'$)$_2$ fragments for tumour imaging in patients. The emission of positrons, negatrons and Auger electrons by $^{64}$Cu may permit combined PET imaging and RIT of pancreatic cancer (i.e. “PET theranostics”). Studies to evaluate the effectiveness of $^{64}$Cu-NOTA-panitumumab F(ab$'$)$_2$ fragments for RIT of pancreatic cancer are planned.

### 2.7 Acknowledgments

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Chapter 3
EGFR-targeted metal chelating polymers (MCPs) harboring multiple pendant PEG$_{2k}$ chains for microPET/CT imaging of patient-derived pancreatic cancer xenografts

The authors Lu Y and Boyle AJ contributed equally to the production of this manuscript as co-first authors.

All synthesis and analysis of the MCPs described herein were performed by Yijie Lu. Supporting information relating to the synthesis and analysis of the MCPs described herein are available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomaterials.6b0049.

All experiments and analyses of data pertaining to the in vitro and in vivo aspects of MCPs in this manuscript were carried out by Amanda Jean Boyle. Patient-derived orthotopic pancreatic mouse models were provided by Ping-Jiang Cao.
3. EGFR-targeted metal chelating polymers (MCPs) harboring multiple pendant PEG$_{2K}$ chains for microPET/CT imaging of patient-derived pancreatic cancer xenografts

3.1 Abstract

In this study, we developed a new generation of metal chelating polymer (MCP) reagents that carry multiple polyethylene glycol (PEG) pendant groups to provide stealth to MCP-based radioimmunoconjugates (RICs). We describe the MCP synthesis for covalent attachment to panitumumab F(ab')$_2$ fragments (pmbF(ab')$_2$) in which different numbers of pendant methoxy-PEG chains [M = 2000, ~ 45 ethylene glycol (EG) repeat units, referred to as PEG$_{2K}$] are incorporated into the polymer backbone. The pendant PEG$_{2K}$ chains were designed to provide a protein-repellent corona so that metal chelators attached closer to the polymer backbone will be less apparent to the physiological environment. DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) groups to chelate $^{64}$Cu were installed on these conjugates to be employed for PET imaging. The conjugation of MCPs to pmbF(ab')$_2$ were based on a UV quantifiable bis-aromatic hydrazone formation under mild conditions (pH 5-6) between an aromatic aldehyde introduced on $\varepsilon$-NH$_2$ groups of lysines in the F(ab')$_2$ fragments and a hydrazinonicotinamide (HyNic) group installed on the initiating end of the MCP. Three MCPs with 17 polyglutamide (PGlu) repeat units, DOTA chelators and with an average of 2, 4 and 8 pendant PEG$_{2K}$ chains were studied to examine their in vitro and in vivo characteristics, as well as their potential for PET/CT imaging. A pmbF(ab')$_2$-MCP conjugate carrying 2 PEG$_{2K}$ and one carrying 8 PEG$_{2K}$ pendant chains in the polymer were selected for microPET/CT imaging and biodistribution studies in tumour-bearing mice. Orthotopic pancreatic PDX tumours were visualized by PET/CT imaging. These RICs showed low levels of liver and spleen uptake along with even lower levels of kidney uptake. These encouraging results confirm the stealth properties of the MCPs with pendant PEG$_{2K}$ chains.

3.2 Introduction

Pancreatic cancer is the 4th leading cause of cancer related deaths worldwide and has the highest mortality rate of all cancers. Pancreatic cancer is an aggressive disease with very few symptoms,
and so patients are often at an advanced stage in disease progression at the time of diagnosis [11]. Surgical resection offers the only opportunity for long-term survival, but less than 20% of patients are candidates for surgical treatment due to local vascular invasion or metastasis. Very limited progress has been made, with a low perioperative mortality rate of 5% [204]. Therefore, there is an urgent need for new diagnostic and therapeutic strategies for pancreatic cancer.

Human epidermal growth factor receptor (EGFR) is a cell-surface receptor for members of the epidermal growth factor family (EGF-family) of extracellular protein ligands and is associated with cancer cell proliferation, invasion, and survival. EGFR is overexpressed in >90% of cases of pancreatic cancer and is the target of the fully human IgG2 monoclonal antibody, Panitumumab (Vectibix; Amgen) [178, 179, 205]. Panitumumab has been approved by the U.S. Food and Drug Administration and studied clinically for treatment of EGFR-positive malignancies [179]. In our initial experiments, we developed a novel radioimmunoconjugate (RIC) that combines $^{64}$Cu Positron Emission Tomography (PET) and Computed Tomography (CT) imaging and radioimmunotherapy (RIT); and so lends itself to the area of PET theranostics [206]. Panitumumab F(ab’)2 fragments (pmabF(ab’)2) were modified with a mean number of ca. 3.2 p-SCN-Bn-NOTA (2-S-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid) which can form a stable complex with the cytotoxic radionuclide $^{64}$Cu [207]. Copper-64 is a radioisotope with a half-life of 12.7 h which decays by positron emissions ($\beta^+\), 0.65 MeV [17.4%], beta emissions ($\beta$, 0.57 MeV [39.0%]), and low energy Auger electrons. Thus, this radioisotope has potential both as a diagnostic PET radioimaging agent, and for radiotherapy [58, 59]. Our results showed that $^{64}$Cu-labeled pmabF(ab’)2 was able to visualize pancreatic cancer patient-derived xenografts (PDX) implanted subcutaneously (s.c.) or orthotopically on the pancreas in non-obese diabetic severe combined immunodeficiency (NOD-scid) mice by PET imaging. However only one in >20 pmabF(ab’)2 was radiolabeled with $^{64}$Cu based on the achieved specific activity (SA) of 3.6 MBq/μg. Thus, a high proportion of EGFR were bound by non-radiolabeled immunoconjugates, limiting the radioimaging quality and the cytotoxic potency.

Antibody-conjugated metal chelating polymers (MCPs) can significantly increase the specific activity of RICs [128, 129]. We have developed several types of functional MCPs in which metal chelators on the pendant groups carry radioisotopes suitable for both imaging and therapy, and
the end group is available for bioconjugation to antibodies or other targeting moieties [131, 133, 208]. First, we used MCPs carrying diethylenetriaminepentaacetic acid (DTPA) to chelate \(^{111}\)In, an Auger emitting radionuclide, and conjugated the MCP to trastuzumab (tmab, Herceptin™). Trastuzumab is widely used for breast cancer treatment due to its specific targeting ability towards human epidermal growth factor receptor-2 (HER2), which is overexpressed on some types of breast cancer cells. To simplify construction of the RIC and to enable us to screen a family of different polymers with different backbones and different chain lengths, a series of polymers, each with a biotin end group were synthesized. In parallel, we derivatized the Fab fragment of tmab (tmFab) with streptavidin (SAv) using a poly(ethylene glycol) (PEG) spacer with 24 ethylene glycol (EG) units (tmFab-SAv) [131, 208]. In this way, we were able to take advantage of the strong and rapid binding between biotin and SAv to construct a family of RICs (tmFab−SAv−MCP complexes). By surface plasmon resonance experiments, we showed that the attachment of the MCP to the tmFab−SAv conjugates had no significant effect on binding to the extracellular domain of HER2, which was characterized by a low nanomolar dissociation constant. Studies in Balb/c mice showed that the tissue distribution of these tmFab−SAv−MCP complexes was sensitive both to the chemical structure of the polymer backbone (polycrylamide, polyaspartamide, or polyglutamide) as well as to the pendant group to which the DTPA units were attached. Then we developed hydrazide-terminated MCPs, which were able to site-specifically conjugate to aldehyde groups generated by sodium periodate oxidation of the pendant glycan in the Fc domain of tmab [133]. These RICs showed highly potent cytotoxic effects against HER2-overexpressed and gene-amplified BC cells and extended their cytotoxicity to cells with intermediate HER2 expression but without gene amplification and to cells that are HER2 overexpressed but trastuzumab-resistant [129].

In the present study, we examine the idea of developing MCP reagents that carry multiple PEG pendant groups to provide stealth to MCP-based RICs. In the studies mentioned above that examined tissue distribution of tmFab−SAv−MCP complexes in Balb/c mice, we found substantial uptake of the conjugates in the liver or kidney, likely associated with negative charges on the pendant groups of the MCPs. Here we describe the synthesis of a new generation of MCPs for covalent attachment to pmabF(ab')\(_2\) in which different numbers of pendant methoxy-PEG chains \([M = 2000, \sim 45\text{ ethylene glycol (EG) repeat units, referred to as PEG}_{2K}\)]
are incorporated into the polymer backbone. In our design, the pendant PEG\textsubscript{2K} chains will provide a protein-repellant (“stealth”) corona so that metal chelators attached closer to the polymer backbone will be less apparent to the physiological environment. Because of the challenges of this new synthesis, we developed and optimized our methodology based upon DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) groups to chelate \textsuperscript{64}Cu in these conjugates to be employed for PET imaging. We are aware of reported problems with leakage of copper ions \textit{in vivo} from the DOTA-copper complex [59, 209]. While the thermodynamic dissociation constant of the Cu\textsuperscript{2+}-DOTA complex is similar to that of the Cu\textsuperscript{2+} complex with NOTA, the Cu\textsuperscript{2+}-NOTA complex is reported to be less susceptible to leakage of copper ions \textit{in vivo} [207]. Because NOTA is so much more expensive than DOTA, we chose DOTA for these initial proof-of-concept experiments, designing our synthesis in such a way that NOTA can be introduced as the metal chelator in the next generation of MCP RICs.

We conjugated MCPs to pmabF(ab’)\textsubscript{2} for labeling with \textsuperscript{64}Cu via UV quantifiable attachment chemistry. This coupling involves the formation of a heat- and pH-stable bis-aromatic hydrazone in a reaction carried out under mild conditions (pH 5-6) between an aromatic aldehyde introduced on \epsilon-NH\textsubscript{2} groups of lysines in the F(ab’)\textsubscript{2} fragments and a hydrazinonicotinamide (HyNic) group installed on the initiating end of the MCP. Three MCPs with 17 polyglutamide (PGlu) repeat units, DOTA chelators and with 2, 4 and 8 pendant PEG\textsubscript{2K} chains were studied to examine their \textit{in vitro} and \textit{in vivo} characteristics, as well as their potential for PET/CT imaging. A pmabF(ab’)\textsubscript{2}-MCP conjugate carrying 2 PEG\textsubscript{2K} and one carrying 8 PEG\textsubscript{2K} pendant chains in the polymer were selected for microPET/CT imaging and biodistribution studies in tumour-bearing mice. Orthotopic pancreatic PDX tumours were visualized by PET/CT imaging. These RICs showed low levels of liver and spleen uptake along with even lower levels of kidney uptake. These encouraging results confirm the stealth properties of the MCPs with pendant PEG\textsubscript{2K} chains.

3.3 Experimental section

3.3.1 Materials

All reagents and solvents, including DTPA (98%, Aldrich) and other compounds were used without further purification unless otherwise noted. Water was purified through a Milli-Q water
purification system (18 MΩ·cm). All other buffers were prepared in our laboratory. Spectra/Pro dialysis membranes (MWCO 1 kDa) were purchased from Spectrum Laboratories, Inc. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM, Acros Organics, 99+%), Millipore Amicon spin filters (15 mL, 3 kDa or 10 kDa MWCO and 4 mL, 3 kDa or 10 kDa MWCO), Vivaspin 20 GE Healthcare MWCO 5k, Amersham ECL Gel Box and Amersham ECL Gel (8-16%) were purchased from Fisher Scientific, Canada. DOTA was purchased from Macrocylics. L-Glutamic acid γ-benzyl ester (BLG), 2-pyridone (2HP), N,N-dimethylformamide (DMF) anhydrous, ethylenediamine (EDA), hydrazine hydrate, picrylsulfonic acid solution 5% (w/v) in H₂O (TNBSA), 1-Pyrenebutyric acid N-hydroxysuccinimide ester (Pyrene NHS), Arsenazo III, DTPA, Gd standard solution and glycine standard solution were purchased from Sigma Aldrich. Experimental details for the syntheses of succinimidyl 4-formyl benzoate (4FB-NHS), succinimidyl 6-hydrazinonicotinate acetone hydrazone (HyNic-NHS) and all polymers are presented in the Supporting Information. PANC-1 pancreatic cancer cells were purchased from American Type Tissue Culture (Manassas, VA, USA) and OCIP23 patient-derived pancreatic cancer cells were provided by the laboratory of Dr. David Hedley (Ontario Cancer Institute, Toronto, ON).

3.3.2 Polymer synthesis and pendant group transformations

Polymer synthesis began with ring-opening polymerization of γ-benzyl-L-glutamate-N-carboxyanhydride (Glu-NCA) initiated by tBoc-PEG-amine ((O-(2-Aminoethyl)-O-[2-(Boc-amino)ethyl]polyethylene glycol Mₙ = 3.5 kDa, Dₚ ≈ 85 (PEG₃.₅K) to yield a tBoc-PEG-poly(γ-benzylglutamate) block copolymer with Dₚ = 17 for the poly(γ-benzylglutamate) (PBLG) block. This polymer was subjected to aminolysis with ethylenediamine to convert the benzyl esters to aminoethyl glutamate groups. Through a series of subsequent modification steps, either DOTA or DTPA metal chelating groups, pyrene as a tracer dye, and PEG₂₅ₐ pendant groups were introduced onto the primary amines to yield three pairs of MCPs with 19 repeat units and with 2, 4 and 8 pendant PEG₂₅ₐ chains. The details of these syntheses and the characterization of the polymers are given in the Supporting Information. Representative ¹H NMR spectra of these polymers are presented in Figures S1–S13 and S17-S19 of the Supporting Information.
3.3.3 Instrumentation

The molecular weights and polydispersities of PBLG samples were measured with a Viscotek GPC Max gel-permeation chromatograph (GPC) equipped with a VE3580 refractive index detector. The analysis was carried out at 80°C at a flow rate of 0.6 mL/min, and the eluent was 1-methyl-2-pyrrolidinone (NMP). The column was calibrated with poly(methyl methacrylate) (PMMA) standards.

The molecular weights and polydispersities of water-soluble samples were measured with a Viscotek size-exclusion chromatograph (SEC) equipped with a Viscotek VE3210 UV/VIS detector, VE3580 refractive index detector, and Viscotek ViscoGEL G4000PWXL and G2500PWXL columns (kept at 30°C). The flow rate was maintained at 1.0 mL/min using a Viscotek VE1122 Solvent Delivery System and VE7510 GPC Degasser. The eluent used was 0.2 M KNO₃, phosphate buffer pH 8.5, 25 mM and 200 ppm NaN₃, and the system was calibrated with poly(methacrylic acid) standards. Polymer samples were dissolved in the eluent prior to injection.

¹H NMR (400 MHz) spectra were recorded on a Varian Mercury 400 spectrometer. All spectra were collected as 128 transients with a delay time of 10 seconds.

SDS-PAGE was performed on an Amersham ECL Gel Box with an Amersham ECL Gel (8-16%). The F(ab')₂ fragments and F(ab')₂ conjugates were stained by PlusOne Coomassie Tablets, PhastGel Blue R-350.

3.3.4 Conjugation of Panitumumab F(ab')₂ with MCPs

3.3.4.1 Modification of Panitumumab F(ab')₂ fragment with 4FB.

A F(ab')₂ solution (1.5 mg, 14 nmol in phosphate buffer 750 μL, pH 8.2, 100 mM containing NaCl 150 mM) was mixed with 4FB-NHS in DMF [10 μL, containing 18 μg (72 nmol) of 4FB-NHS] and the mixture was stirred for 2 h at room temperature. Then the solution was washed with sodium acetate buffer (100 mM, pH 5.0, 6 × 0.40 mL) by a spin filter (MWCO 30 kDa). The concentration of 4FB modified F(ab')₂ was monitored by UV at 280 nm (ε₃50nm = 18,000 M⁻¹cm⁻¹).[210] To perform this measurement, an aliquot of 4FB-
F(ab’)2 solution (27 μL, 20 μM) was added into a sodium acetate buffer (100 mM, pH 5.0, 300 μL) to a final concentration of 2.3 μM. 2-Hydrazinopyridine solution (3.5 μL 50 mM in sodium acetate buffer 100 mM, pH 5.0) was added to a final concentration of 0.5 mM. The UV spectra of the solution were recorded right after the solution of 2-hydrazinopyridine was added into 4FB-F(ab’)2 solution and again after overnight incubation, where we found ΔA350nm = 0.09. Thus the number of 4FB per F(ab’)2 was determined to be ca. 2 (single determination, estimated error ± 10%).

\[
N_{(4FB/F(ab')_2)} = [4FB]/[F(ab')_2] = ΔA_{350nm}/(ε_{350nm}[F(ab')_2])
\]
\[
= 0.09/ (18,000 \text{ M}^{-1}\text{cm}^{-1} \cdot 1 \text{ cm} \cdot 2.3 \text{ μM}) = 2.1
\]

### 3.3.4.2 F(ab’)2-MCP conjugation.

The 4FB-F(ab’)2 solution (0.36 mg, 150 μL for each type of polymer, sodium acetate buffer 100 mM pH 5.0) were then mixed in a UV-cell with a solution of HyNic-modified-MCPs HyNic-PGlu(DOTA)-2PEG2K, HyNic-PGlu(DOTA)-4PEG2K or HyNic-PGlu(DOTA)-8PEG2K, (0.6-0.7 mg, ca. 10 eqv to F(ab’)2) to perform the conjugation. The final concentration of 4FB-F(ab’)2 in each solution was 11 μM with a total volume of ca. 0.30 mL. The UV absorbance at 354 nm of the solution at room temperature was recorded over 4 h, and the reaction was stopped when the number of MCPs per F(ab’)2 was calculated to be approximately one polymer per F(ab’)2, based on ΔA354nm (ε354nm = 29,000 M\(^{-1}\)cm\(^{-1}\)) due to the bis-aryl hydrazone bond formation (See Figure 3.1). We refer to F(ab’)2-MCP conjugates as pmabF(ab’)2-PGlu(DOTA)-nPEG2K (n = 2, 4, 8). We calculated the number of MCPs per F(ab’)2 fragment (\(N_{(MCP/F(ab')_2)}\)) as follows:

\[
N_{(MCP/F(ab')_2)} = \frac{[\text{bis-aryl hydrazone}]/[F(ab')_2]}{ΔA_{354nm}/(ε_{354nm}[F(ab')_2])}
\]

For pmabF(ab’)2-PGlu(DOTA)-2PEG2K

\[
N_{(MCP/F(ab')_2)} = (0.46-0.12)/ (29,000 \text{ M}^{-1}\text{cm}^{-1} \cdot 1 \text{ cm} \cdot 11 \text{ μM}) = 1.1
\]

(2a)

For pmabF(ab’)2-PGlu(DOTA)-4PEG2K

\[
N_{(MCP/F(ab')_2)} = (0.39-0.10)/ (29,000 \text{ M}^{-1}\text{cm}^{-1} \cdot 1 \text{ cm} \cdot 11 \text{ μM}) = 0.9
\]

(2b)

For pmabF(ab’)2-PGlu(DOTA)-8PEG2K

\[
N_{(MCP/F(ab')_2)} = (0.37-0.08)/ (29,000 \text{ M}^{-1}\text{cm}^{-1} \cdot 1 \text{ cm} \cdot 11 \text{ μM}) = 0.9
\]

(2c)

Then the conjugates were analyzed by SDS PAGE.
**Figure 3.1.** Schematic representation of the formation of the bis-aryl-hydrazone-linked immunoconjugates. The F(ab’)_2 fragment was reacted with succinimidyl 4-formylbenzoate (4FB) to attach an aromatic aldehyde to a lysine side chain. An aliquot of the F(ab’)_2 fragment-4FB was then treated with 2-hydrazinopyridine. The hydrazone formed (ε = 18,000 M⁻¹cm⁻¹ at 350 nm) enables determination of the average number of 4FB-linkers introduced on the F(ab’)_2 fragment. The MCP with a HyNic end group was reacted with the 4FB-modified F(ab’)_2 fragment to form the immunoconjugate. The conjugation can be monitored by recording the increase in absorbance at λ = 354 nm (ε = 29,000 M⁻¹cm⁻¹) due to formation of the bis-aryl hydrazone bond (see Figure 3.1).
3.3.5 Radiolabeling with $^{64}\text{Cu}$

$^{64}\text{Cu}$-labeling was achieved by incubating 10 μg of immunoconjugate with 4-37 MBq of $^{64}\text{CuCl}_2$ (Washington University, St. Louis, MO, USA) in 0.1 M sodium acetate buffer (pH 5) for 1 h at 40°C. The final radiochemical purity was measured by instant thin-layer silica gel chromatography (ITLC-SG, Pall Life Sciences, Ann Arbor, MI, USA), developed in 0.1 M sodium citrate (pH 5). The $R_f$ values for the RICs were 0.0, while that of free $^{64}\text{Cu}$ or $^{64}\text{Cu}$-DOTA was 1.0.

3.3.6 EGFR binding of radioimmunoconjugates

The binding of $^{64}\text{Cu}$-labeled pmabF(ab')$_2$-MCPs to EGFR-positive PANC-1 pancreatic cancer cells was measured in single concentration ("one-point") competition binding assays. Approximately 1.5 X 10$^5$ cells were seeded onto wells in a 24-well, flat bottom, tissue culture plate (Sarstedt, Montreal, QC, Canada) and cultured overnight in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose (Gibco Life Technologies; Burlington, ON, Canada) supplemented with 1% penicillin streptomycin and 10% fetal bovine serum (FBS; Invitrogen, Burlington, ON, Canada). Growth medium was removed and cells were incubated with 10 nM of RIC (4 MBq) in 0.5 mL of serum-free medium for 3 h at 4°C. Non-specific binding (NSB) was estimated by repeating the assay in the presence of 100-fold molar excess panitumumab IgG. The medium was removed and adherent cells were rinsed with PBS (pH 7.4) then solubilized in 100 mM NaOH at 37°C for 30 min. The dissolved cells were then transferred to γ-counting tubes, and the total cell-bound radioactivity (TB) was measured in a γ-counter (Model 1480; PerkinElmer, Massachusetts, USA). Specific binding (SB) was calculated by subtracting NSB from TB. EGFR binding was expressed as the percent of TB. The assay was performed in triplicate and the values reported are the mean ± SEM.

3.3.7 Biodistribution studies

The normal tissue biodistribution profiles of $^{64}\text{Cu}$-labeled HyNic-PGlu(DOTA)-2PEG$_{2K}$, HyNic-PGlu(DOTA)-4PEG$_{2K}$, and HyNic-PGlu(DOTA)-8PEG$_{2K}$ were compared in non-tumour bearing Balb/c mice in order to identify major differences in tissue accumulation for MCPs with 2, 4, or 8 PEG$_{2K}$ pendant groups. Groups of 4 mice were injected with 4 MBq (10 μg) of RICs by intravenous (i.v.) tail-vein injection. At 24 h post-injection (p.i.) mice were euthanized by
cervical dislocation under anesthetic [ketamine/xylazine/acepromazine (100 mg/kg; 5 mg/kg; 1 mg/kg)]. Blood and selected tissue samples were collected, weighed, and transferred to γ-counting tubes. Tissue radioactivity was measured in a γ-counter and expressed as the percent injected dose per gram (%ID/g). HyNic-PGlu(DOTA)-2PEG\textsubscript{2K} and HyNic-PGlu(DOTA)-8PEG\textsubscript{2K} were selected for conjugation to pmabF(ab’)\textsubscript{2} in biodistribution and PET/CT imaging studies in tumour-bearing mice. Fragments of PDX OCIP23 tumours were implanted orthotopically on the pancreas of NOD-scid mice via a small incision in the upper left abdomen as previously reported.[211] Tumour-bearing mice were sacrificed at 48 h p.i. of \textsuperscript{64}Cu-labeled pmabF(ab’)\textsubscript{2}-PGlu(DOTA)-8PEG\textsubscript{2K}. The tumour, blood, and selected tissues were collected and weighed, and transferred to γ-counting tubes, then their radioactivity measured on a γ-counter and expressed as %ID/g. All animal studies were conducted under a protocol (#2843.0) approved by the Animal Use Committee at the University Health Network following Canadian Council on Animal Care (CCAC) guidelines.

3.3.8 PET/CT imaging studies

MicroPET/CT was performed at 24 and 48 h post i.v. (tail vein) injection with 20-37 MBq (10 μg) of HyNic-PGlu(DOTA)-2PEG\textsubscript{2K} or \textsuperscript{64}Cu-labeled pmabF(ab’)\textsubscript{2}-PGlu(DOTA)-8PEG\textsubscript{2K} in groups of 4 mice harboring PDX OCIP23 pancreatic cancer xenografts implanted orthotopically on the pancreas. MicroPET imaging was performed on a Focus 220 microPET tomograph (Siemens Preclinical Solutions, Knoxville, TN, USA). Anaesthesia was induced and maintained by 2% isofluorane in O\textsubscript{2}. Images were required for 15-90 min and reconstructed using OSEM, followed by a maximum \textit{a posteriori} probability reconstruction algorithm with no correction for attenuation or partial-volume effects. The full width at half maximum (FWHM) resolution of the microPET tomograph was 1.6 mm. Immediately following microPET imaging, CT was performed on an eXplore Locus Ultra Preclinical CT scanner (GE Healthcare, Mississauga, ON, Canada) with routine acquisition parameters (80 kVp, 70 mA, and voxel size of 150 X 150 X 150 mm). MicroPET and CT images were co-registered using Inveon Research Workplace software (Siemens).
3.3.9 Statistical analysis

The data are represented as the mean ± SEM, n=3-4. Statistical comparisons were performed using an unpaired t-test (p<0.05) using Prism Version 4.0 software (GraphPad).

3.4 Results and Discussion

3.4.1 Design and synthesis of the metal chelating polymers.

Design. In previous studies, we synthesized MCPs based on a PGlu backbone with DTPA-monoamide groups as pendant chelators. These polymers were used to construct RICs carrying $^{111}$In. Some of these polymers had biotin end groups for coupling to a conjugate of SAv with a Fab fragment of trastuzumab. Another polymer had an end group of an adipoylhydrazide for coupling to an aldehyde obtained by oxidation of the glycan of trastuzumab IgG. One of the lessons learned from biodistribution studies of these polymers in mice was that polymers bearing multiple negative charges along the backbone tended to have high levels of normal tissue uptake, particularly by the liver and kidneys. The negative charges arise not only from metal-free DTPA groups that are partially ionized at physiological pH (7.4), but from the fact that the monoamide-DTPA$^{4-}$/In$^{3+}$ chelate has a net negative charge.

For PET imaging studies of pancreatic tumours, we envisioned polymers for conjugation to F(ab’)$_2$ fragments of panitumumab that would carry DOTA-monoamide groups to complex $^{64}$Cu$^{2+}$. The monoamide-DOTA$^{3-}$/Cu$^{2+}$ complex also has a net negative charge. To minimize normal tissue uptake, our polymer design incorporated different numbers of PEG$_{2k}$ pendant groups with $DP_n = 45$ ethylene glycol repeat units. We introduced a long PEG spacer at the initiating end of the polymer to minimize interference with immunoreactivity of the panitumumab F(ab’)$_2$ fragments by the MCP portion of the polymer with its multiple PEG pendant groups. Since the chain-end PEG spacer has a higher degree of polymerization (85) than the PGlu portion (17) carrying the metal chelating and pendant PEG groups, one can think of our target MCPs as diblock copolymers. Another new feature is that we introduced different protein conjugation chemistry (bis-aryl hydrazone formation) that would allow us to monitor the coupling of the metal chelating block copolymer to the F(ab’)$_2$ fragments. To facilitate characterization of these polymers, we synthesized in parallel polymers with DTPA and DOTA
chelators, each attached as a monoamide. The rationale for this extra work will be described below.

**Backbone synthesis and aminolysis.** Polymer synthesis began with ring opening polymerization of $\gamma$-benzyl-\textit{L}-glutamate-\textit{N}-carboxyanhydride (Glu-NCA) in dimethylformamide using tBoc-PEG-amine ((O-(2-Aminoethyl)-O-[2-(Boc-amino)ethyl]polyethylene glycol $M_n = 3.5$ kDa) (PEG3.5K) as the initiator. The EG repeat units of the PEG block play an essential role in the characterization of the polymer by $^1$H NMR through each of the many steps of pendant group transformation. As a consequence, we analyzed the $^1$H NMR of this polymer sample carefully to establish its number average degree of polymerization ($DP_n$) taking advantage not only of the tBoc signal at 1.6 ppm, but also the well resolved signals for the pair of CH$_2$ groups adjacent to the urethane nitrogen and primary amine groups respectively. The spectra are shown in Figure S1, and a discussion of the interpretation of the NMR spectra is presented in Supporting Information (SI). In this way we inferred a value of $DP_n = 85$.

In the ring opening polymerization with this macroinitiator, the initial concentration of \textit{N}-carboxyanhydride was ca. 15 wt% and the reaction was carried out at 0°C for ca. 6 h. A value of $DP_n = 17$ for the PBLG block was determined by $^1$H NMR (Figure S2) by comparing the integrals of signals of the tBoc end protons (1.3-1.5 ppm) to those associated with the polymer pendant benzyl ester groups (6.8-7.2 ppm). This value is lower than expected since the [monomer]/[initiator] ratio was 28. The low conversion may be due to a slow ring opening polymerization reaction rate of Glu-NCA at 0°C. We refer to this polymer as tBoc-PEG$_{85}$-PBLG$_{17}$, where the subscripts refer to the mean degree of polymerization of each block.

A sample of tBoc-PEG$_{85}$-PBLG$_{17}$ polymer in \textit{N}-methylpyrrolidone was treated with excess of EDA in the presence of a catalytic amount of 2-pyridone to convert the benzyl esters to aminoethylamide groups [tBoc-PEG$_{85}$-PGlu(EDA)$_{17}$] a polymer with an -NH$_2$ on each repeat unit. This reaction employed conditions similar to those described previously in which PBLG was subjected to aminolysis with various amines.[212] From the disappearance of the signals of the benzyl group peaks in the $^1$H NMR (Figure S3), we conclude that the conversion of tBoc-PEG$_{85}$-PBLG$_{17}$ (P1) to tBoc-PEG$_{85}$-PGlu(EDA)$_{17}$ (P2) was quantitative. Comparison of the methine proton signal of the PGlu block at (4.2-4.4 ppm) as well as the CH$_2$-NH$_3$ protons at 3.2
ppm, with that of the PEG₈₅ spacer chain (3.7 ppm) indicates that the mean degree of polymerization of the PGlu block was maintained at 17 following the aminolysis reaction. From the peak integration ratio of the tBoc end group (1.4-1.5 ppm) with the methine protons, we find that the tBoc groups also survived the aminolysis reaction.

At this point, a small number of pyrene groups (ca. 0.14 per polymer) were introduced by reaction of the amino groups of the polymer with 1-pyrenebutyric acid N-hydroxysuccinimide ester (pyrene-NHS). The UV signal of the pyrene groups enables detection and quantification of the polymer later in the synthesis. NMR spectra for subsequent steps in pendant group transformation are presented in Figures S4-S13 and S17-S19. Size exclusion chromatograms of these polymers including traces monitored in the UV at the pyrene absorption (345 nm) are shown in supporting information in Figure S14.
Figure 3.2. Synthesis of PEGylated metal chelating block polymers with a HyNic end group for conjugation to F(ab’)2 fragments of panitumumab. The HyNic end group reacts with 4-formylbenzoyl modified F(ab’)2 fragments under mild conditions. Three tBoc-PEG80-polyglutamide MCPs with ca. 17 PGlu repeat units were synthesized with pendant -NH2 groups P2. A small number of pyrene groups (0.14/polymer) were attached as a UV-tracer, followed by PEK2K pendant groups to confer stealth for in vivo studies. The samples bearing an average of 2, 4, and 8 PEG2K chains per polymer are referred to as P3a, P3b, P3c. Note that we do not show the pyrene groups in these structures. The remaining amino groups of the polymers were then converted to DOTA-monoamides (P4a, P4b, P4c) or to DTPA-monoamides (not shown). After deprotection of the tBoc-amino end group of the DOTA polymers, the HyNic group was installed on each chain end.
**Pendant group modification.** We prepared three different polymer samples with different mean numbers of pendant PEG2K chains. Samples of pyrene-labeled tBoc-PEG85-PGlu(EDA)17 in phosphate buffer (50 mM, pH 8) were treated with different amounts of PEG2K-NHS [methoxy-PEG2K-acetic acid N-succinimidyl ester, $M_n = 2000$, 44 ethylene oxide repeat units]. As shown in Figure 3.2, we refer to these three polymers as P3a, P3b, and P3c. The remaining amino groups on the polymer were then used to attach two different types of metal chelators as monoamides, DOTA chelators to obtain polymers P4a, P4b, and P4c or DTPA to obtain polymers P5, P5a, P5b, and P5c. P5 refers to the DTPA polymer with no pendant PEG groups in which all of the amino groups were labeled with DTPA. For these reaction, we employed DMTMM (4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride) as the coupling agent (at pH 8.5) in the presence of excess DOTA or DTPA.

As one can see in the $^1$H NMR spectra presented in Figures S4 - S13, it was not possible to characterize the number of PEG2K pendant chains per MCP or the number of DOTA or DTPA chelators per chain by NMR. The signals of these pendant groups overlapped with those of the PEG85 spacer chain. It was possible, however, by comparing peak integrations of the terminal Boc group at 1.45 ppm to that of the methine peak of the MCP at 4.2 – 4.5 ppm to show that $DP_n$ remained constant at 17. In this way, we infer that the Boc groups persist unchanged during the many pendant group transformation steps for the polymer. Nevertheless, we had to employ a more indirect approach to characterize the pendant groups on the various MCP samples.

Detailed characterization of the number of pendant groups per polymer began with the samples to which the metal chelating groups were attached. Polymer samples P5, P5a, P5b, and P5c were first treated with TFA/water to remove the terminal tBoc group (to form P5’, P5a’, P5b’, and P5c’). Thus these polymers each have one amino group at the initiating end. The molar concentrations of DTPA groups in each sample were determined with the Arsenazo III dye assay using Gd$^{3+}$ as the probe for metal binding. Subsequently, the molar concentrations of amino groups were determined with TNBSA assay. In this assay, the amino groups react with picrylsulfonic acid to form a product with a maximum absorbance in the UV-Vis at 335 nm. Because the pyrene groups also contribute to the absorbance at this wavelength, we monitored the reaction at 400 nm, calibrating the assay with standard solutions of glycine. As a test of the validity of this approach, we examined sample P5’, with no pendant PEG groups (Figure 3.1).
Comparing the results of the two assays, we found \([\text{DTPA}]/[\text{amine}] = 1.26 \text{ mM/73.7 } \mu\text{M} = 17.2\). This result is in good agreement with the value of DP = 17 determined by \(^1\)H NMR of polymer \textbf{P5} in Figure S10 by comparing the integration of the tBoc signals at 1.3-1.5 ppm to that of the backbone methine (4.2-4.4 ppm). The results for polymers \textbf{P5’}, \textbf{P5a’}, \textbf{P5b’}, and \textbf{P5c’} are collected in Table 3.1. From these results, we infer that these polymers contain 2, 4, and 8 PEGs per polymer on average, respectively. Since the DOTA polymers and the DTPA polymers were synthesized from the same precursor polymers \textbf{P3a}, \textbf{P3b}, and \textbf{P3c}, we assume that the corresponding DOTA polymers (\textbf{P4a}, \textbf{P4b}, and \textbf{P4c}), as well as the final polymers HyNic-PGlu(DOTA)-2PEG\(_{2K}\), HyNic-PGlu(DOTA)-4PEG\(_{2K}\) and HyNic-PGlu(DOTA)-8PEG\(_{2K}\), are also characterized by an average of 2, 4, and 8 PEGs per polymer, respectively.

Attempts to repeat the Arsenazo assay on the DOTA polymers were not successful. While the DTPA/Gd complex monitored in the Arsenazo assay forms rapidly (< 1 min), the corresponding DOTA/Gd complex forms slowly (hours) and shows poor reproducibility. Previous results in our laboratory based upon NMR analysis indicated that both DOTA and DTPA undergo near quantitative coupling with pendant amino groups in the presence of DMTMM [213]. Thus we assume that the non-PEGylated pendant groups of the \textbf{P4a}, \textbf{P4b}, and \textbf{P4c} polymers are all substituted with DOTA.
Table 3.1. Molecular weight, hydrodynamic diameters ($d_h$) and percentage recovery of radioactivity from sampled tissues of metal chelating polymer used in this paper.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Polymer</th>
<th>$M_w$ (kDa)$^a$</th>
<th>$&lt;d_h&gt;$ (nm)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1a</td>
<td>HyNic-PGlu(DOTA)-2PEG$_{2K}$</td>
<td>14.8</td>
<td>8.8</td>
</tr>
<tr>
<td>S1b</td>
<td>HyNic-PGlu(DOTA)-4PEG$_{2K}$</td>
<td>17.7</td>
<td>9.2</td>
</tr>
<tr>
<td>S1c</td>
<td>HyNic-PGlu(DOTA)-8PEG$_{2K}$</td>
<td>23.5</td>
<td>10.4</td>
</tr>
</tbody>
</table>

a. The degree of polymerization of the MCPs was obtained by $^1$H NMR. The number of PEG per polymer was characterized based on the TNBSA and Arsenazo III assays are summarized in Table S1.

b. Obtained by Dynamic light scattering. Concentration = 1 mg/mL in PBS.
**End group transformation.** The next step in the synthesis was to introduce a reactive end group to the polymer suitable for bioconjugation. A HyNic end group was installed by incubating the tBoc deprotected MCP with succinimidyl 6-hydrazinonicotinate acetone hydrazone (HyNic-NHS). The degree of end group functionalization was determined in Figure S17-S19 by comparing the integration of $^1$H NMR signals of HyNic end group at 6.9-8.5 ppm to those of the backbone methine (4.2-4.4 ppm), assuming that $DP_n = 17$ for the polymers was unaffected by the reaction. In this way, we found that the HyNic end functionality was ca. 68% for HyNicPGlu(DOTA)-2PEG$_{2K}$, 73% for HyNic-PGlu(DOTA)-4PEG$_{2K}$ and 65% for HyNic-PGlu(DOTA)-8PEG$_{2K}$.

**Bioconjugation.** The MCPs were covalently attached to panitumumab F(ab’)$_2$ via formation of bis-aryl hydrazone (BAH) linkages.[210] The HyNic group at the end of the MCP reacts with a benzaldehyde moiety installed on the F(ab’)$_2$. The extent of bond formation could be monitored by recording the change in absorbance $\Delta A$ at 354 nm, taking advantage of the characteristic UV-vis absorption band of the product at $\lambda = 354$ nm ($\varepsilon_{354}$ nm = 29,000 M$^{-1}$ cm$^{-1}$).[210] This absorbance also allows direct quantification of the mean number of MCPs bound F(ab’)$_2$. This conjugation reaction was carried out in two steps. First F(ab’)$_2$ was incubated with succinimidyl 4-formyl benzoate (4FB-NHS) in phosphate buffer (pH 8.2) to attach benzaldehyde (4FB) groups to the $\varepsilon$-amino groups on lysine residues of the F(ab’)$_2$. The number of 4FB groups per protein can be determined by reacting the modified F(ab’)$_2$ with 2-hydrazinopyridine to form a hydrazone characterized by a UV absorbance at $\lambda_{max} = 350$ nm ($\varepsilon_{350}$ nm = 18,000 M$^{-1}$ cm$^{-1}$).[210] Using a mole ratio of 5 eqv of 4FB-NHS per F(ab’)$_2$, we obtained a product with ca. 2 FB per F(ab’)$_2$. Solutions of this 4FB-F(ab’)$_2$ in a UV cell was treated with an excess of HyNic-PGlu(DOTA)-2PEG$_{2K}$, HyNic-PGlu(DOTA)-4PEG$_{2K}$ or HyNic-PGlu(DOTA)-8PEG$_{2K}$, (total volume 0.3 mL).

Time-dependent UV/Vis-spectra were recorded at room temperature over 4 h, and the reaction was stopped when the change in absorbance ($\Delta A_{354nm}$) corresponded to ca. 1 MCP per F(ab’)$_2$. An example of one such reaction is shown in Figure 3.3a,b. The success of the conjugation reaction was confirmed by SDS-PAGE analysis, which showed an increase in molecular weight compared to the F(ab’)$_2$ fragment itself (Figure 3.3c). The SDS-PAGE gels in Figure 3.3c show several bands at higher molecular weight as well as a band for unreacted F(ab’)$_2$. Since the mean number of polymers per F(ab’)$_2$ is approximately 1.0, we conclude that some of the F(ab’)$_2$ are bound to more than one MCP.
Figure 3.3. Results for conjugation of HyNic end-capped MCPs to 4-formylbenzoyl-functionalized F(ab')₂ fragments of panitumumab. Bis-aryl hydrazone bond formation was followed by UV-Vis measurements, as shown here for formation of F(ab')₂ fragment-MCP-2PEG₂K. In (a) the peak at 345 nm is due to the pyrene on the MCP, whereas the increase in absorbance at the longer wavelengths (ca. 354 nm) is due to hydrazone formation. In (b) we show kinetic plots for conjugation of three HyNic-end-capped MCPs with the 4-formylbenzoyl (4FB)-functionalized panitumumab F(ab')₂ fragments monitored at λ = 354 nm. (c) SDS-PAGE analysis of MCP-conjugated F(ab')₂ fragments. The higher molecular weight bands (arrowheads) compared to the of F(ab')₂ fragments (dashed line) correspond to formation of F(ab')₂-MCP immunoconjugates.
Figure 3.4. Binding of $^{64}$Cu-labeled pmabF(ab')$_2$-MCP-2PEG$_{2K}$, $^{64}$Cu-labeled pmabF(ab')$_2$-MCP-4PEG$_{2K}$, or $^{64}$Cu-labeled pmabF(ab')$_2$-MCP-8PEG$_{2K}$ to EGFR on PANC-1 pancreatic cancer cells was assessed in “one-point” competition binding assays in the presence or absence of 100-fold molar excess panitumumab IgG. Error bars represent the mean ± SEM (n=3).
3.5 EGFR binding of the radioimmunoconjugates

In single concentration (“one-point”) competition binding assays, the binding of $^{64}$Cu-labeled HyNic-pmabF(ab')$_2$-PGLu(DOTA)-2PEG$_{2K}$ (Figure 3.2), $^{64}$Cu-labeled HyNic-pmabF(ab')$_2$-PGLu(DOTA)-4PEG$_{2K}$ and $^{64}$Cu-labeled HyNic-pmabF(ab')$_2$-PGLu(DOTA)-8PEG$_{2K}$ at 10 nM, to PANC-1 cells was reduced to 9.3 ± 0.2%, 52.8 ± 4.2, and 44.0 ± 0.8%, respectively, in the presence of 100-fold molar excess of panitumumab IgG (Figure 3.4). The specific binding of pmabF(ab')$_2$-PGLu(DOTA)-2PEG$_{2K}$ was statistically higher than that of $^{64}$Cu-labeled pmabF(ab')$_2$-PGLu(DOTA)-4PEG$_{2K}$ and pmabF(ab')$_2$-PGLu(DOTA)-8PEG$_{2K}$ ($p<0.005$). The lower degree of specific binding for the conjugates with 4 and 8 pendant PEG$_{2K}$ groups indicates that these conjugates have a higher tendency to bind non-specifically to PANC-1 cells in a way that is not displaced by excess panitumumab IgG. The difference in the extent of non-specific binding of the MCP-conjugate with only 2 PEG$_{2K}$ pendant groups compared to the MCP conjugates with 4 and 8 PEG$_{2K}$ pendant groups may be a result of the increased number of PEG$_{2K}$ pendant groups could interfere with the tertiary structure of the panitumumab F(ab')$_2$ fragments leading to decreased receptor-ligand activity or non-specific binding to cells [214, 215].

3.6 Biodistribution studies

$^{64}$Cu-labeled meal chelating polymers. We first examine the normal tissue distribution at 24 h p.i. of the individual MCPs themselves. $^{64}$Cu-labeled-PGLu(DOTA)-2PEG$_{2K}$, $^{64}$Cu-labeled-PGLu(DOTA)-4PEG$_{2K}$ and $^{64}$Cu-labeled-PGLu(DOTA)-8PEG$_{2K}$ were compared in non-tumour bearing Balb/c mice. These results are presented in Figure 3.5. Table 3.2 shows renal and liver accumulation, as well as the total percent recovery of sampled tissues for PEGylated MCPs compared to a highly charged MCP without PEGylation (Biotin-PAm(DTPA)$_{40}$) from our previous studies [131]. The most striking difference is seen in the renal, liver, and total percentage radioactivity accumulated in mice injected with the highly charged MCP lacking pendant PEG chains which showed up to 13-fold, 7-fold, and 3-fold increase of the PEGylated MCPs of this study for renal, liver, and total radioactivity accumulation, respectively. These results indicate that PEGylation of highly charged MCPs masks the charges in vivo thereby preventing tissue accumulation associated with charge. In comparing the PEGylated MCPs, the polymer with 8 PEG$_{2K}$ pendant chains showed the highest percentage recovery (26.5 ± 3.0%) of radioactivity from
sampled tissues, while the MCPs with 2 and 4 PEG$_{2K}$ pendant chains were significantly lower (7.5 ± 0.7% and 15.3 ± 1.5%, respectively). The most reasonable explanation for these differences is that the extent of renal excretion decreases as the number of pendant PEG chains on the polymer increases. The glomerular filtration cutoff is normally described in terms of the molecular weight of globular proteins (60 kDa [97]). The molecular weights of our polymers range from 19 to 23 kDa. Linear and branched polymers have a random coil shape in solution, and the most important feature of such polymers with respect to glomerular filtration is their hydrodynamic diameter $d_h$. We measured $d_h$ values of our polymers in PBS buffer. These values (Table 3.1) range from 8.8 nm for the polymer with 2 pendant PEG chains to 10.4 nm for the polymer with 8 PEG chains. These solvent-swollen dimensions are 5–10 times greater than that of a globular protein of equivalent molecular weight. While the DOTA groups of metal-free polymers are negatively charged at pH 7.4 (Figure S22) [216], the ionic strength of PBS buffer (with a Debye screening length of ~ 0.7 nm) is high enough to suppress changes in the polymer conformation due to anion repulsion. There are reports in the literature that the hydrodynamic diameter cutoff for kidney filtration is on the order of 6 nm [217]. For example, Duncan et al. [218] studied the effect of molecular weight of N-(2-hydroxypropyl)methacrylamide copolymers on body distribution and rate of excretion in rats. They used eight HPMA copolymer with iodine-125 radiolabeled fractions of narrow polydispersity and mean molecular weight ranging from 12 to 778 kDa, and they found that the molecular weight threshold limiting glomerular filtration was approximately 45 kDa, but they do not report values of $d_h$ for their polymers, Facelli et al [219], also studied the renal clearance of PHPMA, but their samples had a broad polydispersity. They proposed that the renal clearance of PHPMA molecules with large hydrodynamic diameters (up to 131 kDa, $d_h \approx 16$ nm) was due to the deformation of the soft coil structure such that one end of the polymer chain may orient into the pore and penetrate by reptation. This phenomenon has been documented in synthetic systems. Wu et al. [220]. found that when the flux is higher than a certain threshold, a polystyrene coil (6.9 MDa) with a $R_h$ of 100 nm in toluene can pass through a 20 nm pore. Our polymers HyNic-PGlu(DOTA)-2PEG$_{2K}$, HyNic-PGlu(DOTA)-4PEG$_{2K}$, and HyNic-PGlu(DOTA)-8PEG$_{2K}$ are branched, and the extent that they can deform in overall shape is influenced by the number of PEG branches attached to the polymer backbone. Thus, we explain the differences in renal excretion of these three polymers not only in terms of the differences in their hydrodynamic diameters (shown in Figure S23), but also in terms of the extent that they can deform upon passage
through narrow pores. Charged functional groups also play a critical role in the biodistribution in vivo. In our previous study, we found that DTPA functionalized metal chelating polymer with high density of negative charges along the MCP backbone \([\text{Bi-PAm(DTPA)}_{40}]\) is responsible for the high kidney uptake \((34.8 \pm 2.2 \%\text{ID/g})\) and liver uptake \((35.2 \pm 1.9 \%\text{ID/g})\) after 24 hours [131]. Our recent study shows that the liver and kidney uptake could be significantly reduced when the pendant groups of the MCP becomes a zwitterionic upon saturation of the DTPA groups with stable \(\text{In}^{3+}\) ions [132].

There are reports in the literature that the hydrodynamic diameter cutoff for kidney filtration is on the order of 6 nm [217]. For example, Duncan et al. [218] studied the effect of molecular weight of N-(2-hydroxypropyl)methacrylamide copolymers on body distribution and rate of excretion in rats. They used eight HPMA copolymer with iodine-125 radiolabeled fractions of narrow polydispersity and mean molecular weight ranging from 12 to 778 kDa, and they found that the molecular weight threshold limiting glomerular filtration was approximately 45 kDa, but they do not report values of \(d_h\) for their polymers. Facelli et al. [219] also studied the renal clearance of PHPMA, but their samples had a broad polydispersity. They proposed that the renal clearance of PHPMA molecules with large hydrodynamic diameters (up to 131 kDa, \(d_h \approx 16 \text{ nm}\)) was due to the deformation of the soft coil structure such that one end of the polymer chain may orient into the pore and penetrate by reptation. This phenomenon has been documented in synthetic systems. Wu et al. [220] found that when the flux is higher than a certain threshold, a polystyrene coil (6.9 MDa) with a \(R_h\) of 100 nm in toluene can pass through a 20 nm pore. Our polymers HyNic-PGlu(DOTA)-2PEG\(_{2K}\), HyNic-PGlu(DOTA)-4PEG\(_{2K}\), and HyNic-PGlu(DOTA)-8PEG\(_{2K}\) are branched, and the extent that they can deform in overall shape is influenced by the number of PEG branches attached to the polymer backbone. Thus we explain the differences in renal excretion of these three polymers not only in terms of the differences in their hydrodynamic diameters (shown in Figure S23), but also in terms of the extent that they can deform upon passage through narrow pores. Charged functional groups also play a critical role in the biodistribution in vivo. In our previous study, we found that DTPA functionalized metal chelating polymer with high density of negative charges along the MCP backbone \([\text{Bi-PAm(DTPA)}_{40}]\) is responsible for the high kidney uptake \((34.8 \pm 2.2 \%\text{ID/g})\) and liver uptake \((35.2 \pm 1.9 \%\text{ID/g})\) after 24 hours.[131] Our recent study shows that the liver and kidney uptake could be significantly reduced when the pendant
Table 3.2. Total percentage recovery of radioactivity from sampled tissues at 24 h p.i. of metal chelating polymers used in this paper compared to a similarly charged MCP from our previous studies which lacks PEGylation.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Renal Accumulation (%ID/g)</th>
<th>Liver Accumulation (%ID/g)</th>
<th>Percentage Recovery&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HyNic-PGlu(DOTA)-2PEG&lt;sub&gt;2K&lt;/sub&gt;</td>
<td>1.0 ± 0.1</td>
<td>1.8 ± 0.2</td>
<td>7.5 ± 0.7</td>
</tr>
<tr>
<td>HyNic-PGlu(DOTA)-4PEG&lt;sub&gt;2K&lt;/sub&gt;</td>
<td>1.7 ± 0.2</td>
<td>3.1 ± 0.4</td>
<td>15.3 ± 1.5</td>
</tr>
<tr>
<td>HyNic-PGlu(DOTA)-8PEG&lt;sub&gt;2K&lt;/sub&gt;</td>
<td>2.7 ± 0.2</td>
<td>5.3 ± 0.7</td>
<td>26.5 ± 3.0</td>
</tr>
<tr>
<td>Biotin-PAm(DTPA)&lt;sub&gt;40&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34.8 ± 2.2</td>
<td>35.2 ± 1.9</td>
<td>68.6 ± 2.5</td>
</tr>
</tbody>
</table>

Radioactivity recovered from selected organs as a percentage of the injected dose.

Figure 3.5. Normal tissue distribution of $^{64}$Cu-labeled MCPs with increasing numbers of PEG$_{2k}$ pendant chains in Balb/c mice at 24 h p.i. in comparison to $^{111}$In-labeled highly charged MCP without pendant PEG chains from a previous study [131]. Error bars represent mean %ID/g ± SEM, (n=3-4). The extremely statistically significant decrease in liver and kidney accumulation for highly charged PEGylated MCPs indicate that pendant PEG chains mask the charges of MCPs in vivo ($p<0.00001$).
groups of the MCP becomes a zwitterionic upon saturation of the DTPA groups with stable In$^{3+}$ ions [132].

$^{64}$Cu-labeled MCP-F(ab′)$_2$ conjugates. NOD-scid mice bearing patient-derived OCIP23 human pancreatic cancer xenografts, (4.3 $\times$ 10$^5$ EGFR/cell)[206], implanted orthotopically on the pancreas were injected with $^{64}$Cu-labeled pmabF(ab′)$_2$-PGru(DOTA)-2PEG$_{2K}$ or $^{64}$Cu-labeled pmabF(ab′)$_2$-PGru(DOTA)-8PEG$_{2K}$ and yielded tumour accumulations of 7.31 ± 0.23 %ID/g and 4.9 ± 0.5 %ID/g, respectively, at 48 h p. i. (Figure 3.6). Significantly higher tissue accumulation was observed for pmabF(ab′)$_2$-PGlu(DOTA)-2PEG$_{2K}$ compared to pmabF(ab′)$_2$-PGlu(DOTA)-8PEG$_{2K}$ in lungs, liver, spleen, stomach, intestines, and kidneys ($p<0.05$). The increase in RIC accumulation in these tissues may be caused by the 2PEG$_{2K}$ RIC imparting less masking of the negative charges found at the unsaturated chelator sites of the MCP compared to the masking of these charges that results from the 8PEG$_{2K}$ RIC. The total recovery of selected organs for 2PEG$_{2K}$ and 8PEG$_{2K}$ RICs was 41.2% and 31.7%, respectively. However, despite the apparent variation in tumour accumulation the tumour:blood ratios for the 2PEG$_{2K}$ and 8PEG$_{2K}$ conjugated RICs were comparable at 1.7 and 1.5, respectively. Additionally, the blood:total recovery ratio was found to be 0.3 for both of these RICs. These results indicate that each of these RICs is comparable in an orthotopic PDX mouse model.
Figure 3.6. Tissue distribution of $^{64}$Cu-labeled pmab(ab')$_2$-MCP-2PEG$_{2K}$ and pmab(ab')$_2$-MCP-8PEG$_{2K}$ in NOD-scid mice bearing OCIP23 patient-derived pancreatic cancer xenografts implanted orthotopically at 48 h post-injection. Error bars represent mean %ID/g ± SEM (n=4). Significant differences in tissue accumulation for the RICs is indicated by asterisks (***, $p<0.0005$; **, $p<0.005$; *, $p<0.05$).
In previous studies of $^{111}$In-labeled tmAbFab-SA v complexes with biotin-end-capped MCPs in Balb/c mice and in athymic mice bearing SKOV-3 human ovarian cancer xenografts, we found that biodistribution was very sensitive to the chemical nature of the polymer backbone as well as the charge on the pendant groups. For example, a PGlu polymer with 28 DTPA-containing repeat units showed rather low retention of radioactivity in Balb/c mice at 24 h p.i. (22.5 ± 1.5% without $\text{In}^{3+}$ saturation, 15.6 ± 0.8% with $\text{In}^{3+}$ saturation).[131] In these polymers, the DTPA metal chelators were linked to the polymer backbone by a diethylenetriamine (DET) spacer. The middle nitrogen of this group is protonated at neutral pH. Since metal free DTPA monoamide groups have a charge of -2 to -3 at neutral pH, the repeat units not complexed to $^{111}$In$^{3+}$ carry a net negative charge. In contrast, when DTPA is complexed to a trivalent metal ion, the charge on the metal chelate is -1, and with the DET spacer, the pendant group becomes zwitterionic, with no net charge. For these tmAbFab-SA v complexes, at 48 h p.i. in the tumour-bearing athymic mice, tumour uptake was less than 2 %ID/g, comparable to the level of kidney uptake and slightly larger than the level of liver and spleen uptake [132].

In contrast, a tmabFab-SA v complex with a biotin-end-capped polyacrylamide with 40 DTPA-containing repeat units behaved very differently. In our initial publication, we examined a polymer in which the DTPA metal chelators were linked to the polymer backbone by an ethylene diamine spacer. The spacer did not contribute a positive charge to the metal-DTPA pendant groups, and thus, even when saturated with stable In$^{3+}$, these $^{111}$In-labeled tmAbFab-SA v MCP complexes carried a net charge of -1 on each pendant group. At 48 h p.i. in Balb/c mice, these polymers showed a high level of retained radioactivity as a consequence of a very high level of liver uptake [131].

To test the idea that charge associated with the polymer backbone is a key factor affecting the biodistribution profile of MCP-Fab conjugate, we examined a different polyacrylamide MCP with a DET spacer connecting each DTPA to the backbone. When only some of the DTPA groups were bound to metal ions, the polymer was polyanionic, and we found the same poor biodistribution described in the previous paragraph. However, saturation of the chelator groups with nonradioactive trivalent metal ions converted a polyanionic MCP to a zwitterionic polymer. This modification led to much improved biodistribution, including higher blood levels of radioactivity, lower levels of normal tissue uptake and higher tumour uptake [131, 132].
In the experiments reported here, an anionic charge associated with the polymer repeat unit is inevitable, both because of the metal-free DOTA groups, which carry one or two negative charges at physiological pH (see Figure S22), and because the Cu$^{2+}$/(DOTA-monoamide)$^{3-}$ chelate has a net negative charge. For this reason, we designed our MCPs with pendant PEG$_{2K}$ chains, with the idea that they would form an umbrella-like corona to minimize interaction of the polymer backbone with blood proteins and with tissue. Our experiments show that the steric repulsion of the pendant PEG$_{2K}$ chains provided effective stealth properties, resulting in high blood and tumour levels and low levels of uptake into normal tissues.

In future studies we will employ NOTA as the chelating group on MCPs which should result in decreased liver uptake of free $^{64}\text{Cu}$, since NOTA forms a more stable bond with $^{64}\text{Cu}$ than DOTA.[52] Saturation of NOTA-monoamide groups with divalent ions should also lead to a polymer with no net charge along its backbone.
Figure 3.7. Posterior whole-body coronal microPET/CT images at 24 and 48 h p.i. of $^{64}$Cu-labeled pmab(ab')$_2$-MCP-2PEG$_{2K}$ and pmab(ab')$_2$-MCP-8PEG$_{2K}$ in NOD-scid mice bearing OCIP23 patient-derived xenografts implanted orthotopically on the pancreas. Tumours are well visualized (red circles) and the liver is also visible (blue arrow).
3.7 MicroPET/CT imaging studies

MicroPET/CT imaging successfully visualized PDX OCIP23 xenografs implanted orthotopically on the pancreas of NOD-scid mice at 24 and 48 h p.i. with $^{64}$Cu-labeled pmabF(ab’)$_2$-PGLu(DOTA)-2PEG$_{2K}$ or $^{64}$Cu-labeled pmabF(ab’)$_2$-PGLu(DOTA)-8PEG$_{2K}$ (Figure 3.7). In previous studies with $^{64}$Cu-pmabF(ab’)$_2$ without the addition of an MCP, we observed liver metastases in this mouse model [206]. We speculate that we may be visualizing liver metastases in Figure 3.7 since we observe localized visualization of regions within the liver, not exclusively non-localized general accumulation. We also observe an increase in both tumour and liver accumulation for the 8PEG$_{2K}$ RIC from 24 to 48 h p.i., however, we do not see a drastic change in tumour or liver accumulation for the 2PEG$_{2K}$ RIC from 24 to 48 h indicating a more gradual tumour and liver accumulation for 8PEG$_{2K}$ compared to 2PEG$_{2K}$ RIC. The faster accumulation of the 2PEG$_{2K}$ RIC in tumour and liver metastases is likely due to the higher EGFR binding ability of this RIC, as well as the smaller size of 2PEG$_{2K}$ RIC compared to 8PEG$_{2K}$ RIC which would lead to a faster permeation of the tumour than a larger RIC.

3.8 Conclusions

We conjugated MCPs to pmabF(ab’)$_2$ for labeling with $^{64}$Cu via the conjugation of a heat and pH-stable bis-aromatic hydrazone between an aromatic aldehyde introduced on ε-NH$_2$ groups of lysines in the F(ab’)$_2$ fragments and a hydrazinonicotinamide (HyNic) group installed on the initiating end of the MCP. Three MCPs with 17 PGLu repeat units, DOTA chelators and with 2, 4 and 8 pendant PEG chains of M = 2000 (PEG$_{2K}$) were studied to examine their in vitro and in vivo characteristics, as well as their potential for PET/CT imaging. We conclude that excellent stealth of RICs can be achieved by incorporating PEG$_{2K}$ pendant groups into the MCPs and that $^{64}$Cu-labeled pmabF(ab’)$_2$-MCP-2PEG$_{2K}$ and pmabF(ab’)$_2$-MCP-8PEG$_{2K}$ can both successfully visualize orthotopic pancreatic PDXs in NOD-scid mice, and these have comparable biodistribution profiles in vivo. Future studies will investigate a comparison to employing the intact IgG form of panitumumab.

3.9 Acknowledgments

The authors acknowledge support from NSERC Canada as well as by a grant from the Canadian Cancer Society Research Institute (Grant #701682) with funds from the Canadian Cancer
Society. We thank the Spatio-Temporal Targeting and Amplification of Radiation Response (STTARR) program for use of their microPET and CT imaging systems.

### 3.10 Supporting information

Additional experimental details for synthesis and characterization of all polymers (tBoc-PEG3.5k-amine, **P1**, **P2**, **P3a-c**, **P4a-c**, **P5**, **P5a-c**, MCP-2PEG2K, MCP-4PEG2k and MCP-8PEG2K) and 4FB-NHS, HyNic-NHS; representative $^1$H NMR spectra of these compounds; additional results and discussion of characterizing the chain length of the initiator and characterizing the number of PEGs/MCP and SEC profile of **P2**, **P4a-c** with a refractive index detector and UV detector. This material is available free of charge via the Internet at http://pubs.acs.org.
Chapter 4
Radioimmunotherapy with $^{64}$Cu-panitumumab F(ab$'$)$_2$ Fragments Combined with Gemcitabine and PARP Inhibitor in Pancreatic Cancer Xenograft Mouse Models
4 Radioimmunotherapy with $^{64}$Cu-panitumumab F(ab')$_2$ Fragments Combined with Gemcitabine and PARP Inhibitor in Pancreatic Cancer Xenograft Mouse Models

4.1 Abstract

*Introduction:* We examined radioimmunotherapy (RIT) with $^{64}$Cu-panitumumab F(ab')$_2$ fragments alone or combined with radiosensitizing gemcitabine (GEM) and a poly(ADP)ribose polymerase inhibitor (PARPi) in patient-derived pancreatic cancer tumour xenografts (PDX) or PANC-1 pancreatic cancer xenografts in NOD-scid mice.

*Methods:* Normal tissue toxicity was assessed in NOD-scid mice administered i.v. 10 µg $^{64}$Cu-panitumumab F(ab')$_2$ or $^{64}$Cu-anti-mouse EGFR F(ab')$_2$ fragments (1.85-12.85 MBq). Body weight was monitored, and blood was analyzed for complete blood cell counts (CBC) and serum alanine aminotransaminase (ALT) and creatinine (SCr). RIT studies were performed in NOD-scid mice engrafted s.c. with patient-derived OCIP23 pancreatic tumours treated with $^{64}$Cu-panitumumab F(ab')$_2$ fragments (3.7 MBq). Subsequent RIT studies were performed in PANC-1 xenograft mouse models with $^{64}$Cu-panitumumab F(ab')$_2$ fragments (12.95 MBq) alone or in combination with GEM and/or PARPi.

*Results:* No normal tissue toxicity was observed with $^{64}$Cu-panitumumab F(ab')$_2$ or $^{64}$Cu-anti-mouse EGFR F(ab')$_2$ fragments. Treatment with $^{64}$Cu-anti-mouse EGFR F(ab')$_2$ fragments (12.85 MBq) decreased hemoglobin, red blood cell counts, and hematocrit. No change in body weight occurred with any treatment. OCIP23 PDX mice showed a slight tumour growth delay when treated with 3.7 MBq $^{64}$Cu-panitumumab F(ab')$_2$. PANC-1 xenograft mice exhibited no significant differences in median survival between control groups: normal saline, 32.2±6.9 days; GEM + PARPi, 43.5±9.7 days; $^{64}$Cu-panitumumab F(ab')$_2$ fragments, 26.8±14.3 days; and $^{64}$Cu-panitumumab F(ab')$_2$ + GEM, 54.7±20.2 days. Mice treated with $^{64}$Cu-panitumumab F(ab')$_2$ + GEM + PARPi survived longer than all control groups, 69.4 ± 11.7 days, except for $^{64}$Cu-panitumumab F(ab')$_2$ + GEM. Mice treated with $^{64}$Cu-panitumumab F(ab')$_2$ + GEM + PARPi had the greatest tumour growth delay.
Conclusions: RIT with $^{64}\text{Cu}$-panitumumab F(ab')$_2$ fragments delayed tumour growth and prolonged survival in pancreatic cancer mouse models when combined with GEM and PARPi. Evaluation of the normal tissue toxicity of the $^{64}\text{Cu}$-anti-mouse EGFR F(ab')$_2$ fragments suggests that there may not be significant EGFR-mediated toxicity from $^{64}\text{Cu}$-panitumumab F(ab')$_2$ fragments in humans.

Advances in Knowledge and Implications for Patient Care: Combination RIT with $^{64}\text{Cu}$-panitumumab F(ab')$_2$ fragments and radiosensitizing GEM and PARPi may be effective for inhibiting the growth of pancreatic cancer tumours in humans but radionuclides with higher $\beta$-particle abundance would likely be more effective.

4.2 Introduction

Pancreatic cancer is among the most lethal cancers and holds the highest mortality rate among all major cancers, with a 5-year survival rate of 6%, and a 75% mortality rate within the first year after diagnosis [1]. Few early symptoms are associated with pancreatic cancer, and the symptoms remain vague, such as back pain, lethargy, and new onset diabetes [10]. In addition to being nearly asymptomatic, pancreatic cancer is very aggressive, such that patients are often at an advanced stage in disease progression at the time of diagnosis [11]. Additionally, only 10-20% of pancreatic cancer patients are considered candidates for resection due to tumour invasion of major blood vessels or metastases to the liver or other organs [12]. Patients who present as having resectable tumours, are sometimes found to have unresectable and locoregionally metastasized tumours upon surgery [16]. Limited progress has been made in the diagnosis and treatment of this devastating disease and there is an urgent need for new strategies.

Gemcitabine (GEM) has been the first-line monotherapy for pancreatic cancer since the late 1990’s when it was shown to be superior to 5-fluorouracil [19]. GEM has been studied in combination with other chemotherapeutic agents though with limited success [221]. The role of external beam radiation therapy in the treatment of pancreatic cancer is highly controversial mainly due to the high small bowel toxicity associated with its delivery [23]. One potential strategy for harnessing radiation for effective treatment of pancreatic cancer lies in the use of radioisotopes conjugated to monoclonal antibodies (mAbs) which can target receptors overexpressed on pancreatic cancer tumours [radioimmunotherapy (RIT)], thereby
specifically delivering cytotoxic amounts of radioactivity to tumours while sparing normal tissues [42]. Indeed, a Phase I clinical trial has been completed investigating the alpha-emitter $^{212}$Pb conjugated to trastuzumab as a single agent RIT in patients with HER2+ malignancies including pancreatic cancer (ClinicalTrials.gov Identifier: NCT01384253).

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein and first member of the human epidermal growth factor receptor (HER/EGFR/ErBb) family, whose activation triggers many signaling pathways leading to cell proliferation, survival, and invasion [28]. EGFR is present on up to 90% of pancreatic cancer tumours and its overexpression correlates to a more advanced disease stage in pancreatic cancer, demonstrating the potential in targeting this antigen to identify and potentially treat aggressive tumours [28, 29].

Panitumumab F(ab’)2 fragments can be modified with a NOTA chelator for radiolabeling with $^{64}$Cu [206]. $^{64}$Cu (half-life 12.7 h) is a versatile radionuclide which emits positrons ($\beta^+ = 650$ keV (17.4%)) that can be used in PET imaging, as well as $\beta$-particles ($\beta^- = 570$ keV (39.0%)) and low energy Auger electrons that allow RIT. The therapeutic potential of $^{64}$Cu-labeled mAbs and peptides has been demonstrated preclinically in many studies [57-60, 222, 223].

Pancreatic cancer exhibits intrinsic resistance to chemotherapy with GEM, despite this being the first-line therapy [155], and is also resistant to radiation therapy [163]. Yet, GEM has been demonstrated to act as a radiosensitizer for X-irradiation (x-IR) and $\gamma$-irradiation (\(\gamma\)-IR), as well as for RIT with an $^{131}$I-labeled anti-MUC1 mAb against pancreatic cancer tumours [105, 165]. A Phase I clinical trial demonstrated improved survival for patients with locally advanced pancreatic cancer receiving the combination of GEM plus radiotherapy compared to patients receiving GEM alone [167]. A mutation in the p53 tumour suppressor gene is common among pancreatic cancer tumours [224] and the radiosensitizing effect of GEM is independent of p53 [225], so therein lies the merit in employing GEM to enhance RIT of pancreatic cancer. More recently, preclinical studies have combined GEM with a radiosensitizer and/or a GEM-sensitizer, in treatment with $\gamma$-IR and RIT [101, 164, 166, 169, 170]. For example, Al-Ejeh et al. demonstrated that the combination of RIT with $^{177}$Lu-labeled anti-EGFR mAb and chemotherapy with GEM plus a checkpoint kinase 1 inhibitor (CHK1i), PF-477736, caused tumour growth arrest in mice engrafted with PANC-1 pancreatic cancer xenografts [101]. Another combination that has shown potent
radiosensitizing effects preclinically *in vitro* is GEM and rucaparib, a poly ADP ribose polymerase inhibitor (PARPi) [164]. Poly(ADP-ribose)polymerase (PARP) is a DNA repair enzyme that is overexpressed in cancer cells compared to normal cells [226]. The PARPi, rucaparib, has also been studied combined with chemotherapeutic drugs and $^{177}$Lu-labeled anti-EGFR mAb in an EGFR-positive triple negative breast cancer mouse model where the combination demonstrated tumour growth arrest [227].

Our objective in this study was to examine the radiosensitization of pancreatic cancer xenografts in mice to fractionated RIT with $^{64}$Cu-panitumumab F(ab$'$)$_2$ fragments by combination with GEM and the PARPi, rucaparib, in two pancreatic cancer xenograft models: OCIP23 patient-derived tumour xenografts (PDX) implanted subcutaneously (s.c.) or PANC-1 tumours inoculated s.c. on the flank of the mice.

### 4.3 Methods and materials

#### 4.3.1 $^{64}$Cu-panitumumab F(ab$'$)$_2$ synthesis

Panitumumab IgG2 (Vectibix, Amgen) and Ab30 (Abcam, CA), an IgG2 against murine EGFR, were proteolytically cleaved by pepsin digestion to produce F(ab$'$)$_2$ fragments followed by NOTA-derivatization by reaction with $p$-SCN-Bn-NOTA as previously described [206]. $^{64}$Cu-labeling was achieved by incubating 10 μg of the NOTA immunoconjugates with 1.85-12.95 MBq of $^{64}$CuCl$_2$ (Washington University, St. Louis, MO, USA) in 0.1 M sodium acetate buffer (pH 5) for 1 h at 40°C. The final radiochemical purity was measured by instant thin-layer silica gel chromatography (Pall Life Sciences, Ann Arbor, MI, USA) developed in 0.1 M sodium citrate (pH 5). The radiochemical purity was >90% for all RICs.

#### 4.3.2 Mouse models

Two different mouse models were used in this study. In the first tumour model, primary OCIP23 pancreatic tumours expressing EGFR were passaged *in vivo* in NOD-scid mice as previously described [211] then implanted s.c. in NOD-scid mice to establish the OCIP23 PDX mouse model. In the second tumour model, mice were inoculated with PANC-1 human pancreatic adenocarcinoma cells ($3.7 \times 10^5$ EGFR/cell) [206] which had been cultured *in vitro* in Dulbecco’s modified Eagle medium (DMEM) with high glucose (Gibco Life Technologies, ON,
Canada) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (Invitrogen, ON, Canada) maintained at 5% CO₂, 37°C. NOD-scid mice were inoculated s.c. on the right flank with 1 × 10⁷ PANC-1 cells in 100 µL serum-free media. All animal studies were conducted under a protocol (#2843.0) approved by the Animal Use Committee at the University Health Network following Canadian Council on Animal Care (CCAC) guidelines.

4.3.3 Toxicity studies with ⁶⁴Cu-panitumumab F(ab’)₂ fragments

We examined normal tissue toxicity in non-tumour bearing NOD-scid mice (n=4-5). Mice received an intravenous (i.v.) tail vein injection of 10 µg ⁶⁴Cu-panitumumab F(ab’)₂ fragments (1.85-9.25 MBq). Normal tissue toxicity was assessed by monitoring body weight for 14 days at which time mice were anaesthetized by 2% isofluorane in O₂ and blood was collected by cardiac heart puncture followed by cervical dislocation. Blood samples were analyzed for complete blood cell counts (CBC), as well as serum alanine aminotransaminase (ALT) and serum creatinine (SCr) measurements to assess liver and kidney function, respectively. The maximum tolerated dose (MTD) was defined as the maximum amount of ⁶⁴Cu (MBq) conjugated to 10 µg of panitumumab F(ab’)₂ fragments at which no significant difference was observed for CBCs, ALT and SCr levels, as well as no greater than 10% change in body weight.

4.3.4 Toxicity studies with ⁶⁴Cu-anti-mouse EGFR F(ab’)₂ fragments

To address the issue that panitumumab does not recognize murine EGFR [228], and thus toxicity studies of ⁶⁴Cu-panitumumab F(ab’)₂ fragments may not predict the EGFR-mediated toxicity in humans, ⁶⁴Cu-labeled anti-mouse EGFR F(ab’)₂ fragments were examined at the same RIC treatment level as our second RIT study (12.95 MBq, 10µg) in non-tumour bearing NOD-scid mice (n=3). Normal tissue toxicity was assessed as described above.

4.3.5 RIT studies in OCIP23 PDX mouse model

In our first RIT studies, we administered 10 µg of panitumumab F(ab’)₂ fragments at 3.7 MBq in biweekly fractionated doses to examine fractionated delivery of low specific activity ⁶⁴Cu-panitumumab F(ab’)₂ fragments. In our second RIT studies, we administered 10 µg panitumumab F(ab’)₂ fragments at 12.95 MBq of ⁶⁴Cu-panitumumab F(ab’)₂ fragments in biweekly fractionated doses in addition to radiosensitizing agents GEM and PARPi.
Groups of NOD-scid mice (n=10) bearing OCIP23 PDX implanted s.c. on the right flank were injected i.v. (tail-vein) biweekly with $^{64}$Cu-panitumumab F(ab’)$_2$ fragments (3.7 MBq, 10 µg). Control groups included saline and unmodified panitumumab F(ab’)$_2$ fragments (10 µg). Tumour size was measured with calipers and the volume (mm$^3$) was calculated using the formula: $(\text{length} \times \text{width}^2)/2$. Tumour growth was monitored every 3-4 days throughout the study. The tumour growth index (TGI) was determined by dividing the tumour size on a given day by the tumour size on the first day of treatment, the TGI was plotted against time (days) and fitted to an exponential growth curve. The survival benefit was determined by a Kaplan-Meier analysis [229]. Body weight was monitored throughout the study where a loss of >10% was considered a sign of a diarrheic effect.

4.3.6 RIT studies with radiosensitizing agents in PANC-1 xenograft mouse model

We examined a higher fractionated dose of $^{64}$Cu-panitumumab F(ab’)$_2$ fragments in a less aggressive tumour xenograft mouse model of pancreatic cancer. Groups of NOD-scid mice (n=4-8) bearing PANC-1 xenografts inoculated s.c. on the right flank were injected biweekly by i.v. (tail-vein) of $^{64}$Cu-panitumumab F(ab’)$_2$ fragments (12.95 MBq, 10µg) in a dosing cycle that combined GEM and PARPi with RIT. Table 4.1 shows the 10 day dosing schedule and doses of combination therapy where GEM and PARPi were injected intraperitoneally (i.p.) and the cycle was repeated after day 14, as previously described in the literature [101]. Control groups included treatment with A) normal saline, B) GEM + PARPi, C) $^{64}$Cu-panitumumab F(ab’)$_2$ fragments, D) $^{64}$Cu-panitumumab F(ab’)$_2$ fragments + GEM, and E) $^{64}$Cu-panitumumab F(ab’)$_2$ fragments + GEM + PARPi. Tumour growth was monitored every 3-4 days throughout the study. The tumour growth index (TGI) was determined by dividing the tumour size on a given day by the tumour size on the first day of treatment (\). The TGI was plotted against time (days) and fitted to an exponential growth curve. The survival benefit was determined by a Kaplan-Meier analysis [229]. Body weight was monitored throughout the study where a loss of >10% was considered a sign of toxicity. All animal studies were conducted under a protocol (#2843.0) approved by the Animal Use Committee at the University Health Network following Canadian Council on Animal Care (CCAC) guidelines.
4.3.7 Statistical analysis

Data are represented as the mean ± SD. Statistical comparisons were performed using an unpaired t-test with a Bonferroni correction ($p < 0.05$) using Prism Ver 4.0 software (GraphPad).
Table 4.1. Dosing schedule and doses for combination RIT with $^{64}$Cu-panitumumab F(ab$'$)$_2$ fragments, GEM, and PARPi in a single cycle. Cycles repeat after day 14.

<table>
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<td>$^{64}$Cu-panitumumab F(ab$'$)$_2$</td>
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$^a$ First administration of GEM on a given day.

$^b$ Second administration of GEM on a given day.
4.4 Results

4.4.1 Toxicity Studies with $^{64}$Cu-panitumumab F(ab′)$_2$ fragments

The assessment of normal tissue toxicity with 10 µg of $^{64}$Cu-panitumumab F(ab′)$_2$ (1.85, 3.7, and 9.25 MBq) was performed in NOD-scid mice that were monitored for body weight and sacrificed at 14 days post-treatment when analyses were performed on blood samples for CBC, as well as serum ALT and SCr (Figure 4.1). The MTD was not reached at 9.25 MBq. However, there was an indication of impaired liver function as shown by the elevated ALT levels at this dose, and so, for the first RIT study in OCIP23 PDX mice, a fractionated amount of 3.7 MBq $^{64}$Cu-panitumumab F(ab′)$_2$ was administered every 2 weeks.

4.4.2 Toxicity Studies with $^{64}$Cu-anti-mouse EGFR F(ab′)$_2$ fragments

The assessment of normal tissue toxicity at 12.95 MBq of $^{64}$Cu-anti-mouse EGFR F(ab′)$_2$ (10 µg) was performed in NOD-scid (n=3-5) mice by monitoring for body weight, and then at 14 days post-treatment mice were sacrificed and analyses were performed on blood samples for CBC, as well as serum ALT and SCr measurements (Figure 4.1). Significantly decreased levels of hemoglobin (Hb), hematocrit (HCT), and red blood cell counts (RBC) were observed at 12.95 MBq, 10 µg $^{64}$Cu-anti-mouse EGFR F(ab′)$_2$ fragments compared to 9.25 MBq, 10 µg $^{64}$Cu-panitumumab F(ab′)$_2$ fragments. However, Figure 4.2 shows no change in the body weight over 14 days following administration of $^{64}$Cu-anti-mouse EGFR F(ab′)$_2$. Suggesting no generalized toxicity, and no gastrointestinal (GI) toxicity.

4.4.3 RIT studies in OCIP23 PDX mouse model

RIT studies were conducted in OCIP23 PDX mouse models with treatment delivered every 14 days with $^{64}$Cu-panitumumab F(ab′)$_2$ fragments (3.7 MBq, 10 µg) and with control groups: normal saline (untreated), and unmodified panitumumab F(ab′)$_2$ fragments (10 µg). Figure 4.3 shows the tumour growth curves with tumour growth index (TGI) plotted against time (days) then fitted to an exponential growth model. A slight delay in tumour growth was
Figure 4.1. Toxicity studies following i.v. administration of 10 µg of $^{64}$Cu-panitumumab F(ab$'$)$_2$ fragments (1.85 – 9.25 MBq) or 10 µg $^{64}$Cu-anti-mouse EGFR IgG (12.95 MBq) in NOD-scid mice (n=3-5). Blood was analyzed for complete blood cell counts (CBC) and serum alanine aminotransaminase (ALT) and creatinine (SCr).
Figure 4.2. Body weight (g) of NOD-scid mice for 14 days following treatment with 10 µg of \( ^{64} \text{Cu} \)-anti-mouse EGFR (12.95 MBq). Data for individual mice are plotted to demonstrate the effect on individual mice. There is no decrease in body weight, indicating no general toxicity from the treatment.
Figure 4.3. Tumour growth indexes for NOD-scid mice implanted s.c with OCIP23 patient-derived pancreatic cancer tumours then treated with normal saline, panitumumab F(ab’)_2 (10 μg), or ^{64}Cu-panitumumab F(ab’)_2 (3.7 MBq, 10 μg). A slight delay in tumour growth is observed for mice treated with ^{64}Cu-panitumumab F(ab’)_2 fragments. Data represents the mean TGI ± SEM (n=10).
observed for mice treated with $^{64}$Cu-panitumumab F(ab’)$_2$ fragments. The results of this RIT study showed a significant increase in median overall survival for mice treated with panitumumab F(ab’)$_2$, or $^{64}$Cu-panitumumab F(ab’)$_2$ fragments, 23.9 ± 5.8 days survival and 25.0 ± 5.4 days survival post-treatment, compared to the normal saline treated control group which had 17.2 ± 4.6 days survival (Figure 4.4). No significant difference in survival was observed between groups of mice treated with unlabeled compared to $^{64}$Cu-labeled panitumumab F(ab’)$_2$ fragments.

4.4.4 RIT studies with radiosensitizing agents in PANC-1 xenograft mouse model

RIT studies were conducted in NOD-scid mice bearing PANC-1 xenografts with a higher fractionated dose of $^{64}$Cu-panitumumab F(ab’)$_2$ fragments (12.95 MBq, 10 µg) combined with GEM and PARPi. Table 4.1 shows the dosing schedule for this combination therapy and fractionated RIT study in one cycle. Cycles were repeated every 14 days for a total of up to 6 cycles. All treatment groups significantly delayed tumour growth compared to the normal saline treated control group, while GEM + PARPi, $^{64}$Cu-panitumumab F(ab’)$_2$ fragments, and $^{64}$Cu-panitumumab F(ab’)$_2$ fragments + GEM had similar growth curves. A significantly greater tumour growth delay was observed for $^{64}$Cu-panitumumab F(ab’)$_2$ fragments + GEM + PARPi compared to all treatment groups except for $^{64}$Cu-panitumumab F(ab’)$_2$ alone (Figure 4.5F).

No significant improvement in survival was observed between RIT and control groups, but a significant improvement was observed with RIT combined with GEM and PARPi (Figure 4.6). There were no significant differences in median survival found between the control groups A) normal saline; 32.2 ± 6.9 days, B) GEM + PARPi; 43.5 ± 9.7 days, C) $^{64}$Cu-panitumumab F(ab’)$_2$ fragments; 26.8 ± 14.3 days, and D) $^{64}$Cu-panitumumab F(ab’)$_2$ fragments + GEM; 54.7 ± 20.2 days. Mice treated with the full combination of $^{64}$Cu-panitumumab F(ab’)$_2$ fragments + GEM + PARPi had significantly longer median survival than all control groups, at 69.4 ± 11.7 days, except for $^{64}$Cu-panitumumab F(ab’)$_2$ fragments + GEM, at 54.7 ± 20.2 days.
Figure 4.4. Survival data for NOD-scid mice implanted s.c with PDX OCIP23 patient-derived pancreatic cancer tumours then treated with normal saline, panitumumab F(ab')<sub>2</sub> (10 µg), or <sup>64</sup>Cu-panitumumab F(ab')<sub>2</sub> (3.7 MBq, 10 µg). An improvement in survival was noted for mice treated with panitumumab F(ab')<sub>2</sub> and <sup>64</sup>Cu-panitumumab F(ab')<sub>2</sub> compared to normal saline treated control mice but no difference was observed for these two treatments.
Figure 4.5. Tumour growth index curves for NOD-scid mice implanted s.c. with PANC-1 pancreatic cancer xenografts (n=5). Mice were treated with $^{64}$Cu-panitumumab F(ab’)$_2$ fragments (12.95 MBq, 10 µg) on Day 2 of the dosing cycle for up to 6 cycles. GEM was administered by i.p. injection at 1 mg on Day 1, and then twice at 0.15 mg on Days 4, 7, and 10. A PARPi, rucaparib, was administered i.p. at 40 µg on Days 1-5. Individual mice are plotted with initial tumour size indicated in the legend for each treatment group: A) saline, B) RIT, C) RIT + GEM, D) GEM + PARPi, and E) RIT + GEM + PARPi. Comparison of treatment groups is shown in panel F where data represents the mean TGI ± SEM (n=5).
Figure 4.6. Survival data for NOD-scid mice inoculated s.c. with PANC-1 pancreatic cancer xenografts. $^{64}$Cu-panitumumab F(ab$'$)$_2$ fragments were administered i.v. at 12.95 MBq, 10 µg. Mice were treated with normal saline, GEM + PARPi, $^{64}$Cu-panitumumab F(ab$'$)$_2$, $^{64}$Cu-panitumumab F(ab$'$)$_2$ + GEM, or $^{64}$Cu-panitumumab F(ab$'$)$_2$ + GEM + PARPi.
4.5 Discussion

Pancreatic is a very aggressive form of cancer for which patients are often at late stage when diagnosed, with inoperable locoregionally invasive primary tumours or metastatic disease. RIT offers a systemic radiotherapy option that can target pancreatic tumours by binding to receptors that are overexpressed on tumour cells compared to normal tissues. RIT can be enhanced when combined with radiosensitizers and a higher overall dose of RIT can be delivered when treatment is given as multiple fractionated doses [101, 107, 230]. This study investigated the use of low specific activity $^{64}$Cu-panitumumab F(ab′)$_2$ fragments delivered i.v. (tail vein) in fractionated doses alone, or in combination with the radiosensitizers, GEM and the PARPi, rucaparib.

EGFR targeting is appropriate for pancreatic cancer due to its frequently elevated expression in pancreatic cancer tumours, however, there is concern of EGFR mediated toxicity with anti-EGFR therapy since it is also expressed to varying degrees on normal tissues, including liver, kidney, and epidermal tissues. Evaluating the toxicity of panitumumab and RIT aimed at EGFR overexpression utilizing panitumumab in a mouse model is a challenge because panitumumab does not recognize murine EGFR, so mouse models cannot adequately predict toxicity to normal tissues in human subjects. In this study we examined the normal tissue toxicity of an anti-mouse EGFR mAb to predict the toxicity of $^{64}$Cu-labeled anti-EGFR therapy with an IgG$_2$ class antibody analogous to panitumumab. No previous studies have evaluated EGFR mediated toxicities due to the limitation of panitumumab not recognizing murine EGFR. In our toxicity studies, we observed significant decreases in Hb, HCT, and RBC for mice treated with 12.95 MBq, 10 µg of $^{64}$Cu-anti-murine EGFR F(ab′)$_2$ compared to mice treated with 9.25 MBq, 10 µg $^{64}$Cu-panitumumab F(ab′)$_2$. Less than 10% of bone marrow stem cells express EGFR in mice (less than 2% in bone marrow lineage+ and lineage– cells, and <10% in early stage hematopoietic stem cells) [231]. Since there is low EGFR expression in bone marrow stem cells, these changes in Hb, HCT, and RBC indicate mild bone marrow toxicity caused by increased circulation of a murine mAb, and hence increased exposure of bone marrow to the β-emissions from $^{64}$Cu (cross-fire effect), in a mouse model compared to the fully human mAb panitumumab in a mouse model which would clear the blood more quickly. This indicates that if $^{64}$Cu-panitumumab F(ab′)$_2$ fragments were injected into human subjects there may be increased toxicity due to a cross-
fire effect compared to what is observed for $^{64}$Cu-labeled anti-mouse EGFR in preclinical mouse studies due to a potentially longer circulation time in humans. The amount of panitumumab administered is not concerning, considering that we inject 0.5 mg/kg panitumumab F(ab’)$_2$ fragments with each injection, which after performing a dose conversion from mouse to human by dividing by 12.3 [232] represents a dose of 0.04 mg/kg in a human, while the main toxicity (rash) from panitumumab in humans occurs in 100% of patients with 2.5 mg/kg panitumumab [233]. Diarrhea is a toxicity concern for anti-EGFR therapies, such as panitumumab, since EGFR is present on intestinal tissue [234]. Our results show that the amount of 10 µg per 20 g mouse of panitumumab F(ab’)$_2$ does not cause a GI toxic effect, as indicated by no change in body weight with anti-mouse EGFR F(ab’)$_2$. EGFR is moderately expressed in the liver and kidneys and elevated serum ALT and SCr indicate toxicity to each of these organs respectively. We did not observe elevated serum ALT or SCr with $^{64}$Cu-labeled anti-mouse EGFR F(ab’)$_2$ fragments, suggesting that $^{64}$Cu-panitumumab F(ab’)$_2$ may not cause significant toxicity to the liver and kidneys in human subjects when administered at a comparable body weight-adjusted treatment dose (MBq/kg). Indeed, radiolabeled panitumumab has been studied as a PET imaging agent in patients with metastatic colon cancer who were injected with 37 MBq of $^{89}$Zr-panitumumab where no significant toxicity was observed [116].

We performed RIT studies in OCIP23 PDX mouse models where we observed modestly delayed tumour growth in mice treated with $^{64}$Cu-panitumumab F(ab’)$_2$ fragments compared to mice treated with unmodified panitumumab F(ab’)$_2$ fragments. Improved survival was observed for both unmodified and $^{64}$Cu-labeled panitumumab F(ab’)$_2$ fragments compared to untreated mice, with no difference in survival observed between these two treatment groups. These results are representative of the highly aggressive nature of OCIP23 PDX mouse models which grow very quickly and with highly variable tumour growth rates due to variations in hypoxia levels which have previously reported for this mouse model [235]. This caused a large range initial tumour volumes (91 – 772 mm$^3$) as well as mice reaching the humane endpoint due to ulceration of the epidermis at the site of tumour implantation prior to reaching the maximum allowable tumour size endpoint of 15 mm diameter. This result led us to use a less aggressive mouse model for the subsequent RIT studies.

We performed combination therapy studies with $^{64}$Cu-panitumumab F(ab’)$_2$ fragments plus
GEM and a PARPi in the less aggressive PANC-1 xenograft mouse model of pancreatic cancer. The observation of significantly longer survival for mice treated with RIT combined with GEM and PARPi compared to all other treatment groups indicates that these radiosensitizers greatly increase the effectiveness of $^{64}$Cu-panitumumab F(ab′)$_2$ fragments at 12.95 MBq, 10 µg. Preclinical RIT studies using $^{64}$Cu-labeled mAbs have been performed in vivo with higher amounts of radioactivity given as a single injection [57]. In one study, 33 MBq of $^{64}$Cu-mAb directed at Lewis-Y antigen was administered in a mouse model bearing LS174T colorectal xenografts where it modestly delayed tumour growth but was not able to achieve tumour growth arrest [57]. In another study, 74 MBq of a $^{64}$Cu-labeled mAb raised against human colorectal cancer was administered to a hamster model bearing GW39 human colon carcinoma xenografts where only hamsters with small tumours exhibited tumour regression [58]. In our study, we were able to deliver as much as a total of 78 MBq in 12.95 MBq fractionated doses when combined with GEM and PARPi, with no observable signs of toxicity. However, neither our studies nor those described previously in the literature with $^{64}$Cu-labeled mAbs demonstrated tumour growth arrest in all models investigated, leading us to the conclusion that other β-particle emitting radionuclides may be more appropriate for future studies if regression of a solid tumour is the desired primary outcome.

In two separate preclinical studies $^{177}$Lu-anti-EGFR mAbs were administered at 6 MBq per 20 g mouse combined with either radiosensitizing combination GEM + CHK1i [101] or combination chemotherapeutic drugs docetaxel and doxorubicin combined with PARPi [227]. In both studies tumour growth arrest was observed with these combination treatments. In the studies performed here, we administered as much as 12.95 MBq per 20 g mouse as a single dose of $^{64}$Cu-panitumumab F(ab′)$_2$ fragments without observing tumour growth arrest. These results suggest that $^{64}$Cu may not be ideal for RIT, since the β-emissions account for only 39% of decays, despite their moderate energy. For this reason, $^{177}$Lu (half-life 6.7 d) may be a more appropriate radioisotope for RIT applications since it emits moderate energy β-emission [$\beta^- = 498$ keV (78.6%), $\beta^- = 385$ keV (9.1%), and $\beta^- = 176$ keV (12.2%)] in high abundance as well as low abundance γ-emissions [$\gamma = 113$ keV (6.4%) and $\gamma = 208$ keV (11%) that enable imaging by SPECT. With more than double the percentage of β-emissions per decay, $^{177}$Lu may prove more effective in RIT than $^{64}$Cu, despite their having similar β-particle energies at 570 keV for $^{64}$Cu and up to 498 keV for $^{177}$Lu.
4.6 Conclusions

RIT with $^{64}$Cu-panitumumab F(ab’)$_2$ fragments combination with GEM and PARPi significantly delayed tumour growth and improved survival in mice with PANC-1 human pancreatic cancer tumours in. There was no normal tissue toxicity observed during the RIT study at the doses administered, but tumours did continue to grow. Evaluation of $^{64}$Cu-anti-mouse EGFR F(ab’)$_2$ fragments in mouse toxicity studies indicated that EGFR-mediated toxicity was not significant which predicts that $^{64}$Cu-panitumumab F(ab’)$_2$ would be safe in comparable human studies. Future studies investigating the radiobiological effectiveness of $^{64}$Cu- and $^{177}$Lu-panitumumab F(ab’)$_2$ fragments will provide further understanding of the response of pancreatic cancer to $^{64}$Cu- and $^{177}$Lu-RIT.
Chapter 5
Radiobiological Effectiveness of $^{64}$Cu and $^{177}$Lu-NOTA-panitumumab F(ab')$_2$ fragments for Radioimmunotherapy of Pancreatic Cancer Cell Lines
5 Radiobiological Effectiveness of $^{64}$Cu and $^{177}$Lu-NOTA-panitumumab F(ab’)$_2$ fragments for Radioimmunotherapy of Pancreatic Cancer Cell Lines

5.1 Abstract

Introduction: The aim of this study was to determine the relative biological effectiveness (RBE) of $^{64}$Cu- and $^{177}$Lu-panitumumab F(ab’)$_2$ fragments in vitro by comparison to XRT $\gamma$-IR in 4 pancreatic cancer cell lines with varying degrees of EGFR expression, proliferation rates, and radiosensitivity, and one EGFR knockdown pancreatic cancer cell line. By determining the RBE we aimed to investigate the response of these cell lines to $^{64}$Cu-RIT, $^{177}$Lu-RIT, and XRT with $\gamma$-IR by clonogenic survival assays, and $\gamma$-H2AX analysis probing for DNA double-strand breaks based on equivalent radiation doses estimated by cellular dosimetry. We further aimed to correlate the RBE with EGFR density and cell doubling times.

Methods: To study the RBE of $^{64}$Cu- and $^{177}$Lu-labeled panitumumab F(ab’)$_2$ fragments in comparison to XRT $\gamma$-IR in 4 different pancreatic cancer cell lines we first measured EGFR density on AsPC-1, PANC-1, MIAPaCa-2, and Capan-1 pancreatic cancer cells by flow cytometry. Cell doubling time was determined over a 7 days period. Subcellular fractionation studies were performed in cells treated with $^{64}$Cu- or $^{177}$Lu-panitumumab F(ab’)$_2$ fragments (1 MBq/72 nM) for 1, 3, 4, and 24 h. Radioactivity associated with cell surface, cytoplasm, and nucleus source compartments was used to estimate radiation doses to the cell nucleus. Cellular dosimetry was performed based on MCNP modeling to allow amounts of radioactivity incubated with pancreatic cancer cells (MBq) to be converted to absorbed dose (Gy) in clonogenic survival assays. Clonogenic survival assays were performed in all 4 cell lines where cells were treated with XRT $\gamma$-IR (0-8 Gy), $^{64}$Cu- or $^{177}$Lu-RIT (0-8 MBq/72nM) then seeded into 6-well plates and allowed to grow at 37°C, 5% CO$_2$, for 14 days. Then colonies were fixed/stained and counted. The RBE was calculated using the dose required to reduce clonogenic survival to 10% ($D_{10}$) as the measurable effect.

Results: Relative EGFR density was high for AsPC-1 and PANC-1, low for MIAPaCa-2, and moderate for Capan-1 cells. Cell doubling times were 60 h, 33 h, 26 h, and 71 h for AsPC-1, PANC-1, MIAPaca-2, and Capan-1 cells, respectively. Clonogenic survival after $\gamma$-radiation was reduced to 10% ($D_{10}$) for AsPC-1, PANC-1, MIAPaca-2, and Capan-1 cells at 3.2 Gy, 5.0 Gy,
3.7 Gy, and 2.5 Gy. The $D_{10}$ for $^{64}$Cu-RIT was 130 Gy, 13 Gy, 17 Gy, and 192 Gy for AsPC-1, PANC-1, MIAPaCa-2, and Capan-1 cells, respectively. The $D_{10}$ for $^{177}$Lu-RIT was 21 Gy, 26 Gy, 28 Gy, and 36 Gy, for AsPC-1, PANC-1, MIAPaCa-2, and Capan-1 cells, respectively. The associated RBE compared to XRT $\gamma$-IR for $^{177}$Lu-RIT was 0.15, 0.19, 0.13, and 0.07; and for $^{64}$Cu-RIT the RBE was 0.03, 0.38, 0.22, and 0.01, for AsPC-1, PANC-1, MIAPaCa-2, and Capan-1 cells, respectively. Results of $\gamma$-H2AX revealed variation in response of pancreatic cancer cells to $^{64}$Cu- or $^{177}$Lu-RIT.

**Conclusions:** The RBE did not correlate with EGFR density for either $^{64}$Cu- or $^{177}$Lu-RIT. RBE was not dependent on cell doubling time for $^{177}$Lu-RIT, while it was dependent on cell doubling time for $^{64}$Cu-RIT with a greater RBE found for fast proliferating cell lines, PANC-1 and MIAPaCa-2. A higher radiation dose is required for RIT with $^{64}$Cu- or $^{177}$Lu-labeled panitumumab F(ab')$_2$ fragments than XRT $\gamma$-IR to reduce the clonogenic survival of pancreatic cancer cell lines to 10%. Importantly, the trend in radioresponsiveness between pancreatic cancer cell lines is not maintained when comparing XRT $\gamma$-IR and RIT with $^{64}$Cu or $^{177}$Lu-panitumumab F(ab')$_2$ fragments, where PANC-1 was most radioresistant to treatment with XRT and least radioresistant to $^{64}$Cu-RIT, while little variation in response was observed between cell lines following treatment with $^{177}$Lu-RIT.

### 5.2 Introduction

Pancreatic cancer is among the most lethal cancers and holds the highest mortality rate among all major cancers. It is the 4th leading cause of cancer-related death in Canada with a 5-year survival rate of 6%, and a 75% mortality rate within the first year after diagnosis [1]. Pancreatic cancer is nearly asymptomatic and very aggressive, such that patients are often at an advanced stage in disease progression at the time of initial diagnosis [11]. Additionally, only 10-20% of pancreatic cancer patients are considered candidates for resection due to tumour invasion of major blood vessels or metastases to the liver or other organs [12]. Patients who present as having resectable tumours are sometimes found to have unresectable and locoregionally metastasized tumours upon surgery [16]. Limited progress has been made in the diagnosis and treatment of this devastating disease and there is an urgent need for new strategies.

Radioimmunotherapy (RIT) allows tumours to be treated with a systemically-administered, injectable agent by conjugating cytotoxic radionuclides to antibodies that bind to proteins that
are overexpressed on cancer tumours. Pancreatic cancer cells can be targeted using a targeting moiety that binds to human epidermal growth factor receptors (EGFR) which are overexpressed in up to 90% of pancreatic cancer tumours [28, 236, 237]. Panitumumab is a fully human monoclonal antibody (mAb) that we previously modified with NOTA chelators to complex $^{64}$Cu (half-life 12.7 h) to study this radioisotope as a theranostic agent because it emits positrons [$\beta^+ = 650$ keV (17.4%)] that allow positron-emission tomography (PET), as well as $\beta$-particles [$\beta^- = 570$ keV (39.0%) and Auger electrons that can be useful for RIT. We successfully imaged orthotopic patient-derived pancreatic cancer tumours in NOD-scid mice [206]. The potential of $^{64}$Cu-labeled anti-tumour monoclonal antibodies (mAbs) or peptides for RIT of malignancies has been demonstrated by several investigators [57-60]. However, $^{64}$Cu may not be ideal for RIT since the $\beta$-emissions account for only 39% of decays. For example, the $^{64}$Cu-labeled anti-EGFR mAb, cetuximab, has been demonstrated as being similarly effective at delaying tumour growth when administered alone as when combined with cisplatin when mice received 22.2 MBq $^{64}$Cu-cetuximab in RIT studies in p53 wild-type colorectal cancer mouse models [60], however tumour growth arrest was not achieved. $^{177}$Lu (half-life 6.7 d) emits moderate energy $\beta$-emission [$\beta^- = 498$ keV (78.6%), $\beta^- = 385$ keV (9.1%), and $\beta^- = 176$ keV (12.2%)] as well as low abundance $\gamma$-emissions [$\gamma = 113$ keV (6.4%) and $\gamma = 208$ keV (11%). With more than double the percentage of $\beta$-emissions per decay, $^{177}$Lu may prove more effective in RIT of pancreatic cancer than $^{64}$Cu, despite these two radionuclides having similar $\beta$-particle energies. In one study, $^{177}$Lu-panitumumab was found to be more effective than $^{177}$Lu-cetuximab in head and neck tumour mouse models where a single dose of 14.8 MBq had higher tumour uptake with $^{177}$Lu-panitumumab and maintained tumour growth arrest throughout the study while $^{177}$Lu-cetuximab treated tumours began to grow after 15 days [54].

The relative biological effectiveness (RBE) of a radionuclide attached to an anti-EGFR antibody is expected to depend upon the target density (EGFR) on the surface of the cell, on the nuclear uptake for proximity to DNA, cell doubling time, as well as radionuclide dose rate. Since Auger electrons are high-LET with a nanometer range in tissues, these particles should have a higher RBE when deposited in the nucleus compared to low-LET $\beta$-emissions [44]. Since $^{64}$Cu has both $\beta$-emissions and Auger electron emissions it may result a higher RBE than $^{177}$Lu if nuclear transport is achieved. In preclinical studies, the RBE can be determined for a radioimmunoconjugate (RIC) by comparison to external radiation therapy (XRT) with $\gamma$-
irradiation (γ-IR) using the dose required to reduce clonogenic survival to 10% (D_{10}) as the measurable effect for comparison. Cai et al., studied ^{64}\text{Cu}-\text{DOTA}-\text{trastuzumab} in radioresistant MCF-7 breast cancer cells derived from a metastatic site where clonogenic survival curves were obtained for ^{64}\text{Cu}-\text{DOTA}-\text{trastuzumab} and XRT by γ-IR from yielding D_{10} values of 2.8 Gy and 5.4 Gy, respectively corresponding to an RBE of 0.5 with ^{64}\text{Cu}-\text{RIT} [238]. In a similar study, rat pancreatic cancer cells (CA20948) and human carcinoid BON cells were treated with ^{177}\text{Lu}-\text{DOTATE} with the RBE reported as 0.3 [239].

The aim of this research is to determine the RBE of ^{64}\text{Cu}- and ^{177}\text{Lu}-\text{panitumumab} F(ab')_2 fragments in vitro by comparison to XRT γ-IR in 4 pancreatic cancer cell lines with varying degrees of radiosensitivity and EGFR expression, and one EGFR knockdown pancreatic cancer cell line. We investigate the response of these cell lines to ^{64}\text{Cu}-\text{RIT}, ^{177}\text{Lu}-\text{RIT}, and XRT with γ-IR through clonogenic survival assays, subcellular fractionation studies, γ-H2AX analysis probing for DNA double-strand breaks, and cellular dosimetry and examine the RBE correlation to EGFR density, cell doubling time, and nuclear localization.

## 5.3 Methods and materials

### 5.3.1 Cell culture

AsPC-1 cells derived from a metastatic ascites site in a patient with pancreatic cancer (high EGFR expression) [30], PANC-1 human pancreatic adenocarcinoma cells (high EGFR expression) [206], MIAPaCa-2 cells derived from human pancreatic cancer tumour tissue (low EGFR expression) [30], and Capan-1 cells derived from metastatic liver site in a patient with pancreatic cancer (moderate EGFR expression) [240], were purchased from American Type Culture Collection (Manassas, VA, USA). EGFR knockdown (EGFR-KO) cells were derived from PANC-1 pancreatic cancer cells by methods previously described in the literature [241]. Briefly, a single-plasmid CRISPR-Cas9 (pX459) delivery vector (Plasmid #62988, Addgene, USA) containing a single guide RNA targeting EGFR (GATGTTCAATAACTGTGAGG ) was transfected into PANC-1 wild-type cells using Lipofectamine 3000 (Invitrogen, USA). 24 h following transfection, cells were selected using 2 μg/mL puromycin (BioShop, CA) for 48 h. Following selection, cells were expanded for 3 days, and diluted for clonal selection. Expanded clones were tested for EGFR gene editing by tracking insertions and deletions, and finally, protein expression was analyzed by western blot and flow cytometry [241]. All cell lines studied
harbour Kras and p53 mutations [40]. RPMI 1640 medium, Dulbecco’s modified Eagle’s medium (DMEM), Iscove’s modified Dulbecco’s Medium (IMDM), penicillin streptomycin (pen/strep), fetal bovine serum (FBS), and horse serum were purchased from Gibco, Life Technologies (Burlington, ON, CA). AsPC-1 cells were cultured in RPMI supplemented with 1% pen/strep and 10% FBS. PANC-1 cells, both wild-type and EGFR knockdown, were cultured in DMEM supplemented with 1% penicillin/streptomycin and 10% FBS. MIAPaCa-2 cells were cultured in DMEM supplemented with 1% penicillin/streptomycin, 2.5% horse serum, and 10% FBS. Capan-1 cells were cultured in IMDM with a final concentration of 1% penicillin/streptomycin and 20% FBS. Cells were cultured in an atmosphere of 5% CO₂ at 37°C.

5.3.2 Flow cytometry

The EGFR densities for AsPC-1, MIAPaCa-2, Capan-1, and EGFR-KO were estimated by flow cytometry compared to PANC-1 wild-type cells for which our group previously reported a $B_{\text{max}}$ of 3.7 $\times$ $10^5$ EGFR/cell [206]. Briefly, $5 \times 10^5$ cells in 0.5 mL of phosphate buffered saline (PBS) were incubated at 4°C for 60 min with 10 μg of staining solution comprised of panitumumab (Vectibix®; Amgen, Thousand Oaks, CA, USA) conjugated to goat anti-human IgG AlexaFluor 647 (Molecular Probes, Eugene, OR, USA). Control samples were incubated with unconjugated goat anti-human IgG AlexaFluor 647. Mean fluorescence intensity was then determined with FlowJo v10 software (Oregon, USA) collecting 15,000 events per cell line.

5.3.3 Cell doubling time

Cell doubling time was determined for each cell line by seeding cells in triplicate in 6-well plates (Sarstedt, Montreal, QC, Canada) in numbers appropriate to allow room for cells to grow for up to 7 days without exceeding 80% confluency. The number of cells per well was counted on a hemocytometer twice daily for up to 7 days. For accuracy, cells were observed under a microscope during trypsinization (0.3 mL trypsin) to ensure detachment from the plate had occurred, then 0.6 mL of media was added, and a cell scraper applied to the well. The cell suspension was transferred to a 1.5 mL microcentrifuge tube (DiaTEC, Diamed, ON, CA) and the well was rinsed with 0.3 mL 0.9% saline which was added to the suspension. A 5 μL aliquot of the cell suspension was then diluted with 40 μL 0.4% Trypan Blue Stain (Gibco, ON, CA) then counted on a manual hemocytometer (Hausser Scientific, PA, USA). The cell numbers in wells were plotted against time and the curves were fitted to an exponential growth model and
analyzed using Prism Version 4.0 software (GraphPad, San Diego, CA, USA) to determine the doubling time and assess the goodness of fit.

5.3.4 Radioimmunoconjugate synthesis

Panitumumab was digested using pepsin to produce F(ab’)2 fragments followed by NOTA-derivatization by reaction with p-SCN-Bn-NOTA as previously described[206]. ⁶⁴Cu-labeling was achieved by incubating 10 μg of the NOTA immunoconjugate with 4-37 MBq of ⁶⁴CuCl₂ (Washington University, St. Louis, MO, USA) in 0.1 M sodium acetate buffer (pH 5) for 1 h at 40°C. ¹⁷⁷Lu-labeling was achieved by incubating 4-37 MBq of ¹⁷⁷LuCl₃ (PerkinElmer) in 0.1 M HEPES buffer (pH 5.5) for 24 h at 37°C. The final radiochemical purity was measured by instant thin-layer silica gel chromatography (ITLC-SG, Pall Life Sciences, Ann Arbor, MI, USA), developed in 0.1 M sodium citrate (pH 5). The Rᵉ values for the RICs were 0.0, while that of free ⁶⁴Cu or ⁶⁴Cu-DOTA was 1.0.

5.3.5 Subcellular fractionation and cellular dosimetry

Cells were grown overnight in 12-well plates then treated with 1-mL ⁶⁴Cu- or ¹⁷⁷Lu-panitumumab F(ab’)₂ (1 MBq/72 nM) for 1, 3, 4, and 24 h. For each time point an extra well was plated without treatment for cells to be counted in order to calculate the kBq/cell in the treated wells. Following treatment, cells were incubated on ice in stripping buffer (200 mM NaOAc, 500 mM NaCl, pH=2.5) for 10 min to dissociate the cell surface radioactivity. Next, the stripping buffer was collected along with 2 washes with PBS. Cells were then incubated with Nuclei EZ Lysis buffer (Sigma) for 1 h on ice resulting in a suspension of nuclear and cytoplasmic radioactivity. The suspension was centrifuged at 3000 x g for 5 min then the supernatant, comprised of cytoplasmic radioactivity, was collected separately from the nuclear radioactivity within the pellet. The radioactive components of cell surface, cytoplasm, and nuclei were measured separately in a γ-counter. The radioactivity per cell (Bq/s/cell) for each subcellular compartment was calculated then plotted against time and the area under the curve (AUC) for each compartment was determined using the trapezoid rule. The cell doubling time for each cell line was used to determine the effective half-life (t₁/₂ₑ) of radionuclides in each cell line as this would represent the biological half-life (t₁/₂ᵇ). The following equation which incorporates both the physical half-life (t₁/₂ₚ) and biological half-life of radionuclides was used to calculate effective half-life:
\[ t_{1/2e} = \frac{t_{1/2p} \times t_{1/2b}}{t_{1/2p} + t_{1/2b}} \]  

Equation 1

which, in turn, was used to calculate the decay constants (0.693/t_{1/2e}) employed in the trapezoid rule. Cell and nuclear radii (R_C and R_N) were estimated by microscopy and confocal microscopy, respectively, and were used to calculate Snyder values (S values). The AUC of this curve was equal to cumulative dose (\( \bar{A}_s \)), which was multiplied by the S values to give the absorbed dose (Gy) associated with any given MBq amount of radioactivity to the nucleus according to the MIRD schema (Eq. 1).

\[ D = \bar{A}_{CS} \times S_{N\rightarrow CS} + \bar{A}_{Cy} \times S_{N\rightarrow Cy} + \bar{A}_N \times S_{N\rightarrow N} \]  

Equation 2

S values were calculated by Monte Carlo N Particle (MCNP) modeling with the mean monolayer S value representing the sum of self- and cross-dose as previously described [238]. In this manner, the amount of radioactivity applied in clonogenic assays (section 2.6) would be converted from MBq to Gy.

5.3.6 Clonogenic survival assays and relative biological effectiveness

Cells were grown overnight in 24-well plates to 80% confluence then treated with increasing doses (0-8 Gy) of XRT by \( \gamma \)-IR from a \(^{137}\)Cs source (Gammacell 40 Exactor, MDS Nordion Inc., Kanata, ON, Canada) then seeded in increasing numbers into 6-well plates and allowed to grow at 37°C, 5% CO_2, for 14 days. Colonies were then fixed and stained with 1% (w/v) methylene blue/50% ethanol and colonies of >50 cells were counted. Clonogenic survival assays with RIT were performed by treating cells overnight with increasing amounts of \(^{64}\)Cu-panitumumab or \(^{177}\)Lu-panitumumab F(ab')_2 fragments in a final volume of 3 mL media (0-8 MBq/72 nM). The plating efficiency (PE) for each experiment was determined by dividing the number of colonies counted by the number of cells seeded in the well. The survival fraction (SF) for each treatment a cell line received was determined by dividing the PE for a given treatment by the PE for the untreated cells. The amount of radioactivity (MBq) applied for each treatment was converted to absorbed dose to nucleus (Gy) by cellular dosimetry (section 5.3.5) then log SF was plotted against dose (Gy) and fit to a linear quadratic survival curve (Eq. 2).

\[ SF = e^{-(\alpha D + \beta D^2)} \]  

Equation 3
The RBE was determined using the dose required to reduce clonogenic survival to 10% ($D_{10}$) taken from clonogenic survival curves as the measurable effect (Eq. 3).

$$RBE = \frac{D_{XRT}}{D_{RIT}}$$  \hspace{1cm} \text{Equation 4}

An RBE $< 1$ indicated that a greater dose was required to cause the same effect with RIT compared to XRT by γ-IR.

### 5.3.7 DNA double-strand breaks in response to treatment with $^{177}$Lu- or $^{64}$Cu-labeled panitumumab F(ab′)$_2$ fragments

The presence of phosphorylated histone 2A (γ-H2AX) in response to treatment with $^{64}$Cu- or $^{177}$Lu-labeled panitumumab F(ab′)$_2$ fragments was analyzed by immunofluorescence confocal microscopy to assess the presence of DNA double-strand breaks (DSBs) caused by the β-emissions from $^{64}$Cu and $^{177}$Lu, and Auger electrons from $^{64}$Cu. Cells grown overnight on glass coverslips (Ted Pella, CA, USA) in 24-well plates were treated with 300 μL of $^{64}$Cu-panitumumab (4 MBq/72 nM) or $^{177}$Lu-panitumumab F(ab′)$_2$ fragments (2 MBq/72 nM) for 5 h or 20 h at 37°C, 5% CO$_2$. At 5 or 20 h post-treatment, cells were fixed by washing twice with 450 μL 2% paraformaldehyde + 0.5% Triton 100 for 15 min. Cells were then washed twice for 5 min with PBS before being incubated for 15 min with 0.5% Nonidet P-40 at room temperature. This was followed by 2 washes with PBS for 5 min. Cells were then blocked with filtered 2% bovine serum albumin (BSA) in PBS containing 1% donkey serum for 1 h at room temperature. Cells were then incubated overnight at 4°C with 450 μL primary antibody of mouse anti-γ-H2AX, 1/800 in filtered 3% BSA. The following day, cells were washed 4 times with 0.5% BSA + 0.175% tween-20 for 10 min. Cells were then incubated with 450 μL secondary antibody of AlexaFluor 488 donkey anti-mouse IgG, 1/500 in filtered 3% BSA for 45 min at room temperature. Next, cells were washed 4 times with 0.5% BSA + 0.175% tween-20 for 10 min. Finally, coverslips with adhered cells were transferred to a microscope slide (Fisher Scientific, CA) onto a drop of mounting solution containing 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI). The coverslips were sealed onto the microscope slides with dibutyl phthalate.

Fluorescence intensity was measured by confocal microscopy with Zen software version 2.3 SP1 (Zeiss Microscopy, Germany). Data was analyzed using ImageJ 1.51k software (National Institutes of Health, USA) and the relative γ-H2AX foci intensity was determined by dividing the integrated foci density by the area of the nucleus.
5.3.8 Statistical analysis

Data are represented as the mean ± SD or SEM. Statistical comparisons were performed by an unpaired t-test with a Bonferroni correction \((p < 0.05)\) using Prism Version 4.0 software (GraphPad).

5.4 Results

5.4.1 Flow cytometry

The EGFR densities of AsPC-1, MIAPaCa-2, Capan-1, and EGFR-KO cells were estimated by flow cytometry by comparison to the EGFR density previously quantified by radioligand binding assay and reported by our group for PANC-1 \((3.7 \times 10^5 \text{EGFR/cell})[206]\) (Figure 5.1). Based on the mean fluorescence intensity, it was estimated that for AsPC-1, MIAPaCa-2, Capan-1, and EGFR-KO there were approximately \(5 \times 10^5 \text{EGFR/cell}, 8 \times 10^4 \text{EGFR/cell}, 1 \times 10^5 \text{EGFR/cell},\) and \(4 \times 10^3 \text{EGFR/cell},\) respectively.

5.4.2 Cell doubling time

When cell proliferation was fit to an exponential growth curve, as shown in Figure 5.2, the cell doubling times were 60 h, 33 h, 26 h, and 71 h for AsPC-1, PANC-1, MIAPaca-2, and Capan-1 cells, respectively. These values were later used to calculate the effective half-life of radioisotopes in cellular dosimetry analyses (Section 5.3.5).

5.4.3 Subcellular fractionation and cellular dosimetry

The AUC representing the accumulation of \(^{64}\text{Cu}-\) and \(^{177}\text{Lu}\)-panitumumab F(ab′)\(_2\) fragments and their S values in cellular compartments (cell surface, cytoplasm, and nucleus) are shown in Tables 5.1 and 5.2. These values were used to calculate the dose (Gy) delivered to the nucleus from 1 MBq/72 nM treatment. The subcellular accumulation of \(^{64}\text{Cu}\)- and \(^{177}\text{Lu}\) for each cell line is shown in Figures 5.3 and 5.4. The highest total AUC associated with \(^{64}\text{Cu}\)-panitumumab F(ab′)\(_2\) treatment was observed in AsPC-1 (+++) cells with \(39000 \pm 5000 \text{Bq·s/cell}\). EGFR KO had the lowest total AUC of \(^{64}\text{Cu}\)-panitumumab F(ab′) with \(5600 \pm 190 \text{Bq·s/cell}\). For cells treated with \(^{177}\text{Lu}\)-panitumumab F(ab′)\(_2\) fragments the highest total AUC was observed in PANC-1 cells (+++) and the lowest total AUC was observed in AsPC-1 (++++) cells, with \(59000 \pm 4900 \text{Bq·s/cell}\) and \(6100 \pm 170 \text{Bq·s/cell},\) respectively.
Figure 5.1. EGFR-density of A) AsPC-1, B) PANC-1, C) MIAPaCa-2, D) Capan-1, and E) EGFR KO pancreatic cancer cell lines analyzed by flow cytometry. Cells were stained with primary panitumumab human IgG2 antibody conjugated to goat anti-human IgG AlexaFluor647 (blue) or with goat anti-human IgG AlexaFluor647 (red) without incubation with panitumumab immunoconjugates.
Figure 5.2. Cell doubling time was determined by cell counting with a manual hemocytometer twice daily for 7 days. Error bars represent the mean ± SEM (n=5-6).
Table 5.1. Estimate of absorbed dose to nucleus of pancreatic cancer cells in a monolayer exposed to $^{64}$Cu-panitumumab F(ab′)$_2$ fragments (1 MBq/72 nM) for 18 hours.

<table>
<thead>
<tr>
<th>Source</th>
<th>$\bar{A}_s$ (Bq·s)$^a$</th>
<th>S value (Gy/Bq·s)</th>
<th>Dose to N (Gy)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AsPC-1</strong> ($R_c^b = 6.5$ µm, $R_N^c = 4.6$ µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell Surface</td>
<td>1.0 ± 0.2 (X10$^4$)</td>
<td>5.63 $\times$ 10$^{-4}$</td>
<td>5.7 ± 1.2</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>2.5 ± 0.3 (X10$^4$)</td>
<td>6.58 $\times$ 10$^{-4}$</td>
<td>16.6 ± 1.9</td>
</tr>
<tr>
<td>Nucleus</td>
<td>3.4 ± 0.4 (X10$^3$)</td>
<td>1.95 $\times$ 10$^{-3}$</td>
<td>6.7 ± 3.8</td>
</tr>
<tr>
<td>Total</td>
<td>3.8 ± 0.5 (X10$^4$)</td>
<td></td>
<td>29.1 ± 3.87</td>
</tr>
<tr>
<td><strong>PANC-1</strong> ($R_c = 11.5$ µm, $R_N = 6.5$ µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell Surface</td>
<td>5.9 ± 0.3 (10$^3$)</td>
<td>1.17 $\times$ 10$^{-4}$</td>
<td>0.69 ± 0.03</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>5.6 ± 0.1 (10$^3$)</td>
<td>1.34 $\times$ 10$^{-4}$</td>
<td>0.74 ± 0.02</td>
</tr>
<tr>
<td>Nucleus</td>
<td>9.1 ± 0.07 (10$^2$)</td>
<td>4.38 $\times$ 10$^{-4}$</td>
<td>0.40 ± 0.01</td>
</tr>
<tr>
<td>Total</td>
<td>12.4 ± 0.1 (10$^3$)</td>
<td></td>
<td>1.83 ± 0.01</td>
</tr>
<tr>
<td><strong>MIAPaCa-2</strong> ($R_c = 10$ µm, $R_N = 6.5$ µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell Surface</td>
<td>2.1 ± 0.01 (10$^3$)</td>
<td>1.58 $\times$ 10$^{-4}$</td>
<td>0.33 ± 0.001</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>1.2 ± 0.08 (10$^4$)</td>
<td>1.74 $\times$ 10$^{-4}$</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>Nucleus</td>
<td>2.8 ± 0.03 (10$^2$)</td>
<td>4.35 $\times$ 10$^{-4}$</td>
<td>0.12 ± 0.001</td>
</tr>
<tr>
<td>Total</td>
<td>1.4 ± 0.08 (10$^4$)</td>
<td></td>
<td>2.62 ± 0.15</td>
</tr>
<tr>
<td><strong>Capan-1</strong> ($R_c = 3.5$ µm, $R_N = 2.5$ µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell Surface</td>
<td>3.8 ± 1.1 (10$^3$)</td>
<td>8.44 $\times$ 10$^{-4}$</td>
<td>3.2 ± 0.95</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>2.4 ± 1.0 (10$^4$)</td>
<td>1.09 $\times$ 10$^{-3}$</td>
<td>26.5 ± 11.1</td>
</tr>
<tr>
<td>Nucleus</td>
<td>2.4 ± 0.6 (10$^3$)</td>
<td>1.00 $\times$ 10$^{-2}$</td>
<td>24.1 ± 6.0</td>
</tr>
<tr>
<td>Total</td>
<td>3.0 ± 1.0 (10$^4$)</td>
<td></td>
<td>53.8 ± 18.16</td>
</tr>
<tr>
<td><strong>EGFR KO</strong> ($R_c = 11.5$ µm, $R_N = 6.5$ µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell Surface</td>
<td>1.3 ± 0.02 (10$^3$)</td>
<td>1.17 $\times$ 10$^{-4}$</td>
<td>0.15 ± 0.2</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>3.9 ± 0.3 (10$^3$)</td>
<td>1.34 $\times$ 10$^{-4}$</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td>Nucleus</td>
<td>4.0 ± 0.5 (10$^3$)</td>
<td>4.38 $\times$ 10$^{-4}$</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>Total</td>
<td>5.7 ± 0.2 (10$^3$)</td>
<td></td>
<td>0.84 ± 0.04</td>
</tr>
</tbody>
</table>

$^a\bar{A}_s$ = time-integrated radioactivity in source compartment.

$^bR_c$ = radius of cell membrane

$^cR_N$ = radius of nucleus
Table 5.2. Estimate of absorbed dose to nucleus of pancreatic cancer cells in a monolayer exposed to $^{177}$Lu-panitumumab F(ab’)$_2$ fragments (1 MBq/72 nM) for 18 hours.

<table>
<thead>
<tr>
<th>Source</th>
<th>$A_s$ (Bq·s)</th>
<th>$S$ value (Gy/Bq·s)</th>
<th>Dose to N (Gy)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AsPC-1 (R$_c$ = 6.5 µm, R$_N$ = 4.6 µm)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell Surface</td>
<td>3.1 ± 0.1 (10$^3$)</td>
<td>1.03 X 10$^{-3}$</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>1.0 ± 0.07 (10$^3$)</td>
<td>1.15 X 10$^{-3}$</td>
<td>1.2 ± 0.09</td>
</tr>
<tr>
<td>Nucleus</td>
<td>1.9 ± 0.3 (10$^3$)</td>
<td>2.01 X 10$^{-3}$</td>
<td>3.9 ± 0.7</td>
</tr>
<tr>
<td>Total</td>
<td>6.0 ± 0.2 (10$^3$)</td>
<td></td>
<td>8.3 ± 0.5</td>
</tr>
<tr>
<td><strong>PANC-1 (R$_c$ = 11.5 µm, R$_N$ = 6.5 µm)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell Surface</td>
<td>2.4 ± 0.1 (10$^4$)</td>
<td>2.70 X 10$^{-4}$</td>
<td>6.4 ± 0.4</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>4.9 ± 0.03 (10$^3$)</td>
<td>3.2 X 10$^{-4}$</td>
<td>1.6 ± 0.01</td>
</tr>
<tr>
<td>Nucleus</td>
<td>3.0 ± 0.3 (10$^3$)</td>
<td>7.6 X 10$^{-4}$</td>
<td>23.2 ± 2.7</td>
</tr>
<tr>
<td>Total</td>
<td>5.9 ± 0.5 (10$^3$)</td>
<td></td>
<td>31.1 ± 3.0</td>
</tr>
<tr>
<td><strong>MIAPaCa-2 (R$_c$ = 10 µm, R$_N$ = 6.5 µm)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell Surface</td>
<td>6.5 ± 1.2 (10$^3$)</td>
<td>2.13 X 10$^{-4}$</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>1.2 ± 0.2 (10$^3$)</td>
<td>2.22 X 10$^{-4}$</td>
<td>0.3 ± 0.04</td>
</tr>
<tr>
<td>Nucleus</td>
<td>3.4 ± 0.2 (10$^3$)</td>
<td>6.69 X 10$^{-4}$</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>Total</td>
<td>11.1 ± 0.2 (10$^3$)</td>
<td></td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td><strong>Capan-1 (R$_c$ =3.5 µm, R$_N$ = 2.5 µm)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell Surface</td>
<td>3.9 ± 0.9 (10$^3$)</td>
<td>4.24 X 10$^{-3}$</td>
<td>16.8 ± 4.0</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>1.3 ± 0.2 (10$^3$)</td>
<td>4.77 X 10$^{-3}$</td>
<td>6.2 ± 0.7</td>
</tr>
<tr>
<td>Nucleus</td>
<td>1.7 ± 0.3 (10$^3$)</td>
<td>8.41 X 10$^{-3}$</td>
<td>14.4 ± 2.9</td>
</tr>
<tr>
<td>Total</td>
<td>6.9 ± 0.7 (10$^3$)</td>
<td></td>
<td>37.4 ± 1.8</td>
</tr>
<tr>
<td><strong>EGFR KO (R$_c$ = 11.5 µm, R$_N$ = 6.5 µm)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell Surface</td>
<td>2.2 ± 0.3 (10$^3$)</td>
<td>2.7 X 10$^{-4}$</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>3.9 ± 0.3 (10$^3$)</td>
<td>3.2 X 10$^{-4}$</td>
<td>0.1 ± 0.01</td>
</tr>
<tr>
<td>Nucleus</td>
<td>2.3 ± 0.4 (10$^3$)</td>
<td>7.6 X 10$^{-4}$</td>
<td>17.7 ± 2.9</td>
</tr>
<tr>
<td>Total</td>
<td>2.5 ± 0.4 (10$^4$)</td>
<td></td>
<td>18.4 ± 2.9</td>
</tr>
</tbody>
</table>

$A_s$ = time-integrated radioactivity in source compartment

$R_c$ = radius of cell membrane

$R_N$ = radius of nucleus
Figure 5.3. Subcellular uptake of radioactivity in cells incubated with $^{64}$Cu-panitumumab F(ab')$_2$ fragments (1 MBq/72 nM) in A) AsPC-1, B) PANC-1, C) MIAPaCa-2, D) Capan-1, and E) PANC-1 KO pancreatic cancer cells over time (h). Cellular compartments measured include cell surface, cytoplasmic compartment, and nuclear compartment. Error bars represent the SD for the mean kBq/cell.
Figure 5.4. Subcellular uptake of radioactivity in cells incubated with $^{177}$Lu-panitumumab F(ab')$_2$ fragments (1 MBq/72 nM) in A) AsPC-1, B) PANC-1, C) MIAPaCa-2, D) Capan-1, and E) EGFR KO pancreatic cancer cells over time (h). Cellular compartments measured include cell surface, cytoplasmic compartment, and nuclear compartment. Error bars represent the SD for the mean kBq/cell.
The highest dose (Gy) to the nucleus from treatment with 1 MBq/72 nM $^{64}$Cu-panitumumab F(ab')$_2$ fragments was observed for AsPC-1 and Capan-1 cells, which did not differ significantly from each other (Table 5.1). Following treatment with 1 MBq/72 nM $^{177}$Lu-panitumumab F(ab')$_2$ fragments the highest dose (Gy) to the nucleus was observed for PANC-1 and Capan-1 cells, which did not differ significantly from each other (Table 5.2).

5.4.4 Clonogenic survival assays and relative biological effectiveness

The clonogenic survival of AsPC-1, PANC-1, MIAPaCa-2, Capan-1, and EGFR KO cells exposed to XRT with γ-IR or to $^{64}$Cu- or $^{177}$Lu-panitumumab F(ab')$_2$ fragments (0-8 MBq/72 nM) was determined. The dose deposited to the nucleus of AsPC-1, PANC-1, MIAPaCa-2, Capan-1, and EGFR KO cells at the same concentration (72 nM) of $^{64}$Cu- or $^{177}$Lu-panitumumab F(ab')$_2$ fragments but different specific radioactivities (0-8 MBq/72 nM) was estimated by cellular dosimetry with MCNP modeling (section 5.3). Figure 5.5 shows the SF plotted against the calculated absorbed dose to the nucleus (Gy) and fit to a linear quadratic equation with different $\alpha$, $\beta$, and $r^2$ values associated with each cell line and treatment (Table 5.3). The dose required to reduce the SF to 10% ($D_{10}$) was determined from each clonogenic survival curve and used to calculate the RBE for each cell line in response to treatment with $^{64}$Cu- or $^{177}$Lu-panitumumab F(ab')$_2$ fragments, the results of which are presented in Table 5.4.

5.4.5 γ-H2AX activity in response to treatment with $^{64}$Cu or $^{177}$Lu-labeled panitumumab F(ab')$_2$ fragments

The presence of γ-H2AX in the nucleus of pancreatic cancer cells treated with $^{64}$Cu or $^{177}$Lu-labeled panitumumab F(ab')$_2$ fragments (4 MBq/72 nM and 2 MBq/72 nM, respectively) was visualized by immunofluorescence confocal microscopy to assess the degree of DNA DSBs after incubating cells for 5 h or 20 h with RIT (Figure 5.6). In these studies, differences in the amount of DNA DSBs were assessed at 5 h and 20 h due to the cell lines having different cell doubling times, and thus spending different amounts of time in the various phases of the cell cycle, including G1 phase, early S phase, late S phase, and G2 phase where DNA DSB repair can occur through homologous recombination or non-homologous end-joining [242]. We also expected differences due to the different dose rates of $^{64}$Cu and $^{177}$Lu due to their different half-lives, 12.7 h and 6.7 days, respectively. No significant differences in DNA DSBs were observed between cells treated with $^{64}$Cu-panitumumab F(ab')$_2$ fragments for 5 hr and the control untreated samples.
(Figure 5.7). After 20 h incubation with $^{64}$Cu-panitumumab F(ab’)$_2$ fragments, DNA DSBs were significantly higher than control untreated samples in AsPC-1 and MIAPaCa-2 cell lines with relative γ-H2AX foci intensity of 1.64 ± 0.2 and 2.76 ± 0.1, respectively, ($p<0.05$). Significantly higher relative γ-H2AX foci intensity was observed at 20 h compared to 5 h treatment for MIAPaCa-2 cells and Capan-1 cells treated with $^{64}$Cu-panitumumab F(ab’)$_2$ fragments, which were found to be 3-fold and 2-fold higher, respectively, ($p<0.05$). In PANC-1 cells treated with $^{177}$Lu-panitumumab F(ab’)$_2$ fragments, the 5 h treatment had 3-fold higher relative γ-H2AX foci intensity, 3.85 ± 0.65, than both the control untreated sample, 1.00 ± 0.21, and the 20 h treatment group, 1.34 ± 0.24. MIAPaCa-2 cells treated for 20 h with $^{177}$Lu-panitumumab F(ab’)$_2$ fragments had 2-fold higher relative γ-H2AX foci intensity, 2.07 ± 0.29, compared to control samples, 1.00 ± 0.08, ($p<0.05$), (Figure 5.7). Capan-1 cells treated with $^{177}$Lu-panitumumab F(ab’)$_2$ fragments for 5 h and 20 h had 2-3 fold higher relative γ-H2AX foci intensity, 2.97 ± 0.57 and 2.40 ± 0.23, respectively, compared to control samples, 1.00 ± 0.31, ($p<0.05$), (Figure 5.7).
Figure 5.5. Clonogenic survival curves for AsPC-1, PANC-1, MIAPaca-2, Capan-1, and PANC-1 EGFR KO pancreatic cancer cell lines treated with increasing doses of A) XRT γ-irradiation, B) $^{64}$Cu-panitumumab F(ab')$_2$ fragments, and C) $^{177}$Lu-panitumumab F(ab')$_2$. The dose (Gy) for RIT was determined from data collected for subcellular fractionation studies. Error bars represent SD of the mean survival fraction (n=3).
Table 5.3. Summary of $\alpha$ (Gy$^{-1}$) and $\beta$ (Gy$^{-1}$) values applied to clonogenic survival curves acquired for AsPC-1, PANC-1, MIAPaCa-2, Capan-1, and EGFR KO pancreatic cancer cell lines in response to XRT with $\gamma$-IR or RIT with $^{64}$Cu- or $^{177}$Lu-panitumumab F(ab$'$)$_2$ fragments.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>XRT $\alpha$</th>
<th>XRT $\beta$</th>
<th>$^{64}$Cu-panitumumab F(ab$'$)$_2$ $\alpha$</th>
<th>$^{64}$Cu-panitumumab F(ab$'$)$_2$ $\beta$</th>
<th>$^{177}$Lu-panitumumab F(ab$'$)$_2$ $\alpha$</th>
<th>$^{177}$Lu-panitumumab F(ab$'$)$_2$ $\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AsPC-1</td>
<td>0.587</td>
<td>0.04</td>
<td>0.003</td>
<td>$1 \times 10^{-4}$</td>
<td>0.08</td>
<td>0.00118</td>
</tr>
<tr>
<td>PANC-1</td>
<td>0.24</td>
<td>0.04705</td>
<td>0.1485</td>
<td>0.002</td>
<td>0.08</td>
<td>0.0002</td>
</tr>
<tr>
<td>MIAPaCa-2</td>
<td>0.3095</td>
<td>0.07341</td>
<td>0.03</td>
<td>0.006</td>
<td>0.06</td>
<td>0.0008</td>
</tr>
<tr>
<td>Capan-1</td>
<td>0.8853</td>
<td>0.01</td>
<td>0.0048</td>
<td>$3.5 \times 10^{-5}$</td>
<td>0.04</td>
<td>0.0006</td>
</tr>
<tr>
<td>EGFR KO</td>
<td>0.13</td>
<td>0.03</td>
<td>0.00987</td>
<td>$1 \times 10^{-5}$</td>
<td>0.02</td>
<td>0.0002</td>
</tr>
</tbody>
</table>
Table 5.4. Summary of experimental results including EGFR density, $D_{10}$ values, and RBE values.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>EGFR Density</th>
<th>$D_{10}$ for XRT</th>
<th>$D_{10}$ for $^{64}$Cu-RIT</th>
<th>RBE for $^{64}$Cu-RIT</th>
<th>$D_{10}$ for $^{177}$Lu-RIT</th>
<th>RBE for $^{177}$Lu-RIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>AsPC-1</td>
<td>5 X $10^5$</td>
<td>3.2</td>
<td>130</td>
<td>0.03</td>
<td>21</td>
<td>0.15</td>
</tr>
<tr>
<td>PANC-1</td>
<td>3.7 X $10^5$</td>
<td>5.0</td>
<td>13</td>
<td>0.38</td>
<td>26</td>
<td>0.2</td>
</tr>
<tr>
<td>MIAPaCa-2</td>
<td>8 X $10^4$</td>
<td>3.7</td>
<td>17</td>
<td>0.22</td>
<td>28</td>
<td>0.13</td>
</tr>
<tr>
<td>Capan-1</td>
<td>1 X $10^5$</td>
<td>2.5</td>
<td>192</td>
<td>0.01</td>
<td>36</td>
<td>0.07</td>
</tr>
<tr>
<td>EGFR KO</td>
<td>4 X $10^3$</td>
<td>6.8</td>
<td>193</td>
<td>0.03</td>
<td>68</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>MEAN:</strong></td>
<td>3.6</td>
<td>0.2</td>
<td>0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.6. Sites of unrepaired DNA double-strand breaks (DSBs) following treatment with A) $^{177}\text{Lu}$-panitumunab F(ab')$_2$ fragments (2 MBq/72 nM) or B) $^{64}\text{Cu}$-panitumunab F(ab')$_2$ fragments (4 MBq/72 nM) visualized by confocal immunofluorescence microscopy for γ-H2AX (green foci) in the nuclei which were stained with DAPI (blue) for AsPC-1, PANC-1, MIAPaCa-2, and Capan-1 pancreatic cancer cell lines.
Figure 5.7. Relative intensity of γ-H2AX foci normalized to cells treated with unlabeled panitumumab F(ab’)_2 fragments for pancreatic cancer cells treated for 5 h or 20 h with A) ^{64}\text{Cu}-panitumumab F(ab’)_2 fragments (4 MBq/72 nM) or B) ^{177}\text{Lu}-panitumumab F(ab’)_2 fragments (2 MBq/72 nM). Values shown represent the mean ± SEM (n=3-5). Significant differences (p<0.05) in relative γ-H2AX foci intensity between control samples and treated samples and between 5 h and 20 h treatment are indicated by asterisks.
5.5 Discussion

In this study we determined the RBE of β-emitting radioisotopes $^{64}$Cu and $^{177}$Lu conjugated to panitumumab F(ab′)$_2$ fragments by comparison to XRT with γ-IR. $^{64}$Cu and $^{177}$Lu have comparable β-particle energies at 570 keV for $^{64}$Cu and up to 498 keV for $^{177}$Lu, however, differences arise in that $^{64}$Cu emits high-LET Auger electrons, has a lower abundance of β-emissions than $^{177}$Lu (39% vs 90%), and a higher dose rate than $^{177}$Lu due to its shorter half-life (12.7 h vs 6.7 days). $^{64}$Cu and $^{177}$Lu have been demonstrated as having therapeutic potential in RIT in vitro and in vivo [57-60, 101, 227, 238, 243], but a direct comparison between these two β-emitters within the same in vitro RIT study has yet to be explored in the literature. Cellular dosimetry allowed us to examine the radiosensitivity of 4 different pancreatic cancer cell lines to RIT with $^{64}$Cu- or $^{177}$Lu-panitumumab F(ab′)$_2$ fragments by converting MBq amounts of RIT treatment to dose to nucleus (Gy), thereby allowing comparison to XRT with γ-IR for RBE determination.

We examined 4 cell lines with different levels of EGFR overexpression, and one EGFR KO cell line. We found no correlation between the degree of EGFR overexpression and the responsiveness of pancreatic cancer cell lines to $^{64}$Cu-panitumumab F(ab′)$_2$ fragments, in that the cell line with the highest EGFR density, AsPC-1, had a 10-fold lower RBE than PANC-1 cells which had comparably high EGFR density. On the same note, AsPC-1 cells (EGFR ++++) exhibited a similar RBE in response to treatment with $^{177}$Lu-panitumumab F(ab′)$_2$ fragments to that with the lowest EGFR density, MIAPaCa-2 (EGFR +). The EGFR KO cell line, derived from PANC-1 wild-type cells, had a 10-fold decrease in response compared to PANC-1 cells following treatment with $^{64}$Cu-panitumumab F(ab′)$_2$ fragments and a 2-fold decrease following treatment with $^{177}$Lu-panitumumab F(ab′)$_2$ fragments, indicating that EGFR positivity is required for response to anti-EGFR RIT, but the degree of response cannot be predicted based on the level of EGFR overexpression when comparing between different cell lines, at least within the range of EGFR expression for the cell lines studied.

Our findings demonstrated that fast proliferating cell lines, PANC-1 and MiaPaCa-2, were more responsive to $^{64}$Cu-RIT than slow proliferating cell lines, AsPC-1 and Capan-1. This finding is supported in the literature where an RBE of 0.5 was reported in response to $^{64}$Cu-DOTA-
trastuzumab in HER2-positive, fast proliferating, MCF-7 breast cancer cells [238] which have a 24 h doubling [244]. Pancreatic cancer cell lines are resistant to radiation therapy to varying degrees, some exhibiting very high levels of resistance [155, 164]. Porcelli et al. investigated the magnitude and variability of pancreatic cancer cell response to γ-IR and found PANC-1 cells to be more radioresistant than MiaPaCA-2, Capan-1, and AsPC-1 pancreatic cancer cells. In these studies, in vitro cell proliferation of PANC-1 could not be reduced below 70% with doses as high as 10 Gy, while the proliferation of MiaPaCA-2, Capan-1, and AsPC-1 were reduced to 10-30% [164]. Our results agree with this study in that the D10 from treatment with XRT γ-IR was higher for PANC-1 cells than for AsPC-1, MIAPaCa-2, or Capan-1. Our results show that this trend does not persist for RIT with 64Cu- or 177Lu-panitumumab F(ab′)2 fragments in that PANC-1 cells were more radiosensitive to both of these RICs than the other cell lines investigated herein.

We performed subcellular fractionation studies to allow cellular dosimetry analysis and convert MBq quantities of radioactivity to dose (Gy) delivered to the nucleus. These studies also allowed us to examine whether there is a correlation between response to RIT and nuclear accumulation of the RIC. Figure 5.1 shows there was no correlation between nuclear accumulation and RBE in response to 64Cu-RIT which emits Auger electrons and β-particles; the highest nuclear accumulation with 64Cu-panitumumab F(ab′)2 fragments occurred in AsPC-1 and Capan-1 cell lines which were the most resistant to treatment. Furthermore, the highest nuclear accumulation of 177Lu-panitumumab F(ab′)2 fragments occurred in PANC-1 and EGFR KO cells; PANC-1 had the highest RBE in response to 177Lu-RIT, but only marginally higher than AsPC-1, MIAPaCa-2, and Capan-1 cell lines which had drastically lower nuclear accumulation of the 177Lu-RIC. EGFR KO cell lines did exhibit a higher RBE than Capan-1 cells in response to 177Lu-RIT, however, Capan-1 cells are BRCA2 mutated and, as such, do not have efficient DNA repair mechanisms [245]. The lack of significant difference between the nuclear accumulations of PANC-1 cells compared to EGFR KO cells indicates a nuclear import mechanism of RIC unrelated to EGFR. This may be an indication of loss of the radioisotope from the 177Lu-RIC, but because the nuclear accumulation in both PANC-1 and EGFR KO were significantly higher than all other cell lines, we cannot conclude that this explains this observation. Additionally, the stability of 177Lu-NOTA has been demonstrated previously in the literature [55, 246]. Residual EGFR occurs on our EGFR KO cells which may allow the mechanism which leads to high nuclear accumulation in PANC-1 wild-type cells to remain intact. EGFR KO cells had
significantly lower accumulation of radioactivity when incubated with $^{177}$Lu-panitumumab F(ab’)$_2$ fragments on the cell surface compared to all other cell lines, indicating that the EGFR KO was an adequate control, however, the observation of greater nuclear uptake in EGFR KO cells compared to all cell lines except its PANC-1 wild-type indicates that there are mechanistic nuclear transportation differences that occur when comparing different cell lines. Indeed, different patterns of internalization of anti-EGFR nanoparticles in PANC-1 and AsPC-1 cells have been reported in the literature, despite these cell lines having similar EGFR density levels [247, 248]. In this study the authors suggest that the difference in nuclear uptake may be due to PANC-1 being derived from a primary tumour while AsPC-1 is derived from a metastatic site, however, our results do not support this theory since MIAPaCa-2 is derived from a primary tumour and did not demonstrate a high nuclear accumulation of the RIC, but rather a low accumulation comparable to the metastatic site-derived cell lines AsPC-1 and Capan-1.

An *in vitro* preclinical study was published in rat pancreatic cancer cells (CA20948) and human carcinoid BON cells comparing high-LET $^{213}$Bi-DOTATATE, an $\alpha$-emitter, to $^{177}$Lu-DOTATATE in which clonogenic survival curves were obtained with each RIC and compared to XRT from a $^{137}$Cs source [239]. The authors determined the RBE using $D_{10}$ as the measurable effect for comparison, and observed an RBE of 2.0 for $^{213}$Bi-DOTATATE and 0.3 for $^{177}$Lu-DOTATATE [239]. In these clonogenic studies the cells were exposed to RICs for 1 h, whereas in our studies, the cells were incubated for 18 h. Interestingly, the RBEs determined for $^{177}$Lu in our study, Table 5.4, are comparable to that observed by these authors, despite the differences in incubation time. This indicates that the incubation time is not critical for a comparable cytotoxic effect to occur in pancreatic cancer cells. In another study of similar design as that described herein, clonogenic survival assays were performed on Capan-2 human pancreatic cancer cells, comparing $^{213}$Bi-DOTATOC and $^{177}$Lu-DOTATOC, in which the authors report RBEs of 3.4 and 1.0, respectively [249]. In both of these studies the authors conclude that $^{213}$Bi-RIT is more effective than $^{177}$Lu-RIT, however, this conclusion is based solely on the RBEs observed *in vitro* without the context of a tumour microenvironment and a 3-dimensional tumour, and an *in vivo* dosimetry study would need to be performed to draw this conclusion. In a study comparing high-LET $^{227}$Th-trastuzumab, an $\alpha$-emitter, and low-LET $^{177}$Lu-trastuzumab targeting HER2 in mice bearing SKBR-3 xenografts, the authors found the RBE to be higher for $^{227}$Th-trastuzumab when these RICs were compared at the same therapeutic effect of 100% prolonged tumour growth
delay compared to control [66]. Yet the therapeutic index was greater for $^{177}$Lu-trastuzumab as determined when the RICs were compared at the same temporary decrease in white blood cell count of 50% decrease compared to control, for which $^{177}$Lu-trastuzumab had 3 times longer growth delay [66]. It is important to perform RBE studies in tumour bearing mice to ascertain an RBE that reflects an in vivo environment where both effects on the tumour and normal tissues may be compared.

We performed γ-H2AX analysis to examine DNA DSBs after 5 h and 20 h treatment with either $^{64}$Cu-panitumumab F(ab')$_2$ fragments (4 MBq/72 nM) or $^{177}$Lu-panitumumab F(ab')$_2$ fragments (2 MBq/72 nM). From cellular dosimetry we observed that the MBq quantities of radioactivity incubated with the cells translated into different doses (Gy) being delivered to the nucleus in each cell line, we observe variation in the induction of γ-H2AX which supports our cellular dosimetry results that different doses (Gy) are delivered to the nucleus in different cell lines when treated with the same MBq amount of radioactivity, as is seen in Figure 5.6 and 5.7. For PANC-1 and Capan-1 cells treatment with 2 MBq/72 nM $^{177}$Lu-panitumumab F(ab')$_2$ fragments corresponded with a dose >60 Gy. This is reflected in PANC-1 cells and Capan-1 cells exhibiting the highest DNA DSB induction at 5 h that was repaired after 20 h incubation with $^{177}$Lu-RIT. Similarly, 2 MBq/72 nM $^{177}$Lu-panitumumab F(ab')$_2$ fragments corresponded to a dose to the nucleus of <20 Gy to AsPC-1 and MIAPaCa-2 cells, which is reflected in these cell lines having very low DNA DSB induction compared to PANC-1 and Capan-1 cell lines. A trend in response of cell lines to $^{64}$Cu-panitumumab F(ab')$_2$ fragments was not observed at the incubation times studied. It is possible that for the fast dose rate of $^{64}$Cu (12.7 h half-life) the time points studied were not adequately short to observe the induction in DNA DSBs and their subsequent repair. It is important to acknowledge that comparing γ-H2AX induction between cell lines is challenging in that cell lines differ in cell doubling time, and hence, differences in the opportunity for cells to repair damage occur, leading to differences in γ-H2AX recruitment.

5.6 Conclusion

In conclusion, fast proliferating cancer cells are more responsive to $^{64}$Cu-RIT than slow proliferating cells, and $^{64}$Cu-RIT is more effective than $^{177}$Lu-RIT in fast proliferating cells in vitro. Pancreatic cancer cell lines respond similarly to $^{177}$Lu-panitumumab F(ab')$_2$ fragments despite variation in EGFR expression and cell proliferation rates. A higher dose of RIT with
$^{64}$Cu- or $^{177}$Lu-labeled panitumumab F(ab')$_2$ fragments than XRT $\gamma$-IR is required to reduce the clonogenic survival of pancreatic cancer cell lines to 10%. Importantly, the trend in radioresponsiveness between pancreatic cancer cell lines is not maintained when comparing XRT $\gamma$-IR and RIT with $^{64}$Cu or $^{177}$Lu-panitumumab F(ab')$_2$ fragments.
Chapter 6
Overall Conclusions
6 Overall conclusions

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

1) $^{64}\text{Cu}$-labeled panitumumab in its Fab, F(ab’)$_2$, and intact IgG forms were developed and characterized for assessment of tumour and normal tissue biodistribution, and microPET/CT imaging demonstrated potential for detecting tumours in a clinically relevant patient-derived orthotopic xenograft mouse model.

2) Three MCPs were developed with 17 polyglutamide (PGLu) repeat units, 30 DOTA chelators and with an average of 2, 4, and 8 pendant PEG$_{2K}$ chains and these were conjugated to panitumumab F(ab’)$_2$ fragments and radiolabeled with $^{64}\text{Cu}$, and examined for their in vitro and in vivo characteristics. Successful visualization of patient-derived orthotopic pancreatic cancer xenografts in mice by microPET/CT imaging was achieved.

3) Normal tissue toxicity and therapeutic efficacy of $^{64}\text{Cu}$-panitumumab F(ab’)$_2$ fragments combined with radiosensitizing chemotherapeutic drugs in vivo in pancreatic cancer xenograft mouse models was studied to examine the therapeutic potential of $^{64}\text{Cu}$ as a PET theranostic agent. Radiosensitization of pancreatic cells in response to RIT with $^{64}\text{Cu}$-panitumumab F(ab’)$_2$ fragments was achieved.

4) $^{64}\text{Cu}$- and $^{177}\text{Lu}$-panitumumab F(ab’)$_2$ fragments were examined in vitro compared to external radiation therapy (XRT) using $\gamma$-irradiation ($\gamma$-IR) in 4 pancreatic cancer cell lines that were characterized for their varying degrees of radiosensitivity, EGFR expression, and proliferation rates. The RBE was not correlated to EGFR density.

5) To examine $^{64}\text{Cu}$- and $^{177}\text{Lu}$-panitumumab F(ab’)$_2$ fragments in vitro compared to external radiation therapy (XRT) with $\gamma$-irradiation ($\gamma$-IR) in 4 pancreatic cancer cell lines with varying degrees of radiosensitivity and EGFR expression, to determine the RBE correlation to EGFR density, cell doubling time, and nuclear localization as described in Chapter 5.
6.1 Summary of key findings

Chapter 2 described the development and characterization, in vitro and in vivo, of $^{64}$Cu-labeled panitumumab in its Fab, F(ab')$_2$, and intact IgG antibody forms. F(ab')$_2$ fragments of panitumumab were produced by proteolytic digestion of panitumumab IgG or non-specific human IgG, purified by ultrafiltration then modified with NOTA chelators for complexing $^{64}$Cu. Panitumumab F(ab')$_2$ fragments were produced in high purity (>90%), derivatized with 3.2 ± 0.7 NOTA/ F(ab')$_2$, and labeled with $^{64}$Cu (0.3-3.6 MBq/μg). Panitumumab IgG and Fab fragments were similarly labeled with $^{64}$Cu. EGFR immunoreactivity was determined in competition and direct (saturation) cell binding assays. The binding of $^{64}$Cu-panitumumab F(ab')$_2$ to OCIP23 or PANC-1 cells was decreased significantly by an excess of panitumumab IgG. The $K_d$ for binding of $^{64}$Cu-NOTA-panitumumab F(ab')$_2$ to EGFR on PANC-1 cells was 0.14 ± 0.05 nM. The in vivo biodistribution of $^{64}$Cu-labeled panitumumab IgG, F(ab')$_2$ and Fab were compared in non-tumour-bearing mice, where F(ab')$_2$ fragments exhibited more suitable normal tissue distribution for tumour imaging with $^{64}$Cu than panitumumab IgG or Fab. F(ab')$_2$ fragments of panitumumab demonstrated lower renal accumulation than Fab fragments and intact IgG, as well as the greatest accumulation in blood. F(ab')$_2$ fragments of panitumumab were used for in vivo biodistribution and microPET/CT studies performed in NOD-scid mice engrafted s.c. or orthotopically with patient-derived OCIP23 pancreatic tumours, or in athymic mice with s.c. PANC-1 human pancreatic cancer xenografts. Tumour uptake at 48 h p.i. of $^{64}$Cu-panitumumab F(ab')$_2$ was 12.0 ± 0.9% injected dose/g (ID/g) in s.c. and 11.8 ± 0.9% ID/g in orthotopic OCIP23 tumours vs. 6.1 ± 1.1% ID/g in s.c. PANC-1 xenografts. Tumour/blood ratios were 5:1 to 9:1 for OCIP23 and 2.4:1 for PANC-1 tumours. Tumour uptake of $^{64}$Cu-non-specific F(ab')$_2$ in OCIP23 xenografts was 5-fold lower than $^{64}$Cu-panitumumab F(ab')$_2$. All tumour xenografts were clearly imaged by microPET/CT at 24 or 48 h p.i. of $^{64}$Cu-panitumumab F(ab')$_2$. This chapter demonstrated that $^{64}$Cu-panitumumab F(ab')$_2$ fragments bound with high affinity to EGFR on pancreatic cancer cells in vitro and localized specifically in patient-derived pancreatic cancer xenografts in mice in vivo, allowing tumour visualization by microPET/CT at 24 or 48 h p.i., thereby demonstrating that EGFR-positive patient-derived pancreatic cancer xenografts in mice can be imaged by microPET/CT.

Chapter 3 examined $^{64}$Cu-labeled MCPs with 19 repeat units and different numbers of pendant PEG chains (2, 4, and 8 PEG$_{2K}$ pendant chains) linked to panitumumab F(ab')$_2$ fragments for
their *in vitro* and *in vivo* characteristics and for PET/CT. MCPs allow high specific activity radiolabeling to amplify the sensitivity for tumour imaging and potency for RIT. The objective of Chapter 3 was to examine how different numbers of pendant PEG chains affect the biodistribution properties of MCPs linked to panitumumab F(ab′)₂ fragments *in vivo*. First, unconjugated ⁶⁴Cu-MCPs were injected i.v. into non-tumour bearing BALB/c mice and biodistribution studies were performed at 48 h p.i to assess the effect of the number of PEG₂K pendant chains per MCP on blood retention and normal tissue uptake. The organ uptake of unconjugated ⁶⁴Cu-labeled MCPs in BALB/c mice at 48 h increased with increasing numbers of PEG₂K pendant chains. Then MCPs conjugated to panitumumab F(ab′)₂ fragments via bis-aromatic hydrazone formation were assessed for EGFR immunoreactivity in 1-point (concentration) binding assays on PANC-1 pancreatic cancer cells where the proportion of specific binding *in vitro* to EGFR for panitumumab F(ab′)₂ fragments linked to MCPs with 2, 4, or 8 PEG₂K pendant chains was 90.7 ± 0.2%, 47.2 ± 4.2%, and 56.0 ± 0.8%, respectively. Panitumumab F(ab′)₂ fragments conjugated to MCPs with 2 PEG₂K pendant chains were selected for studies in tumour-bearing mice due to their high immunoreactivity for EGFR. Despite lower EGFR binding, panitumumab F(ab′)₂ fragments conjugated to MCPs with 8 PEG₂K pendant chains were also selected for studies in tumour-bearing mice due to its longer blood residence time which should maximize tumour uptake. MicroPET/CT imaging and biodistribution studies were performed at 48 h p.i in NOD-scid mice engrafted orthotopically with patient-derived OCIP23 pancreatic tumours. Uptake in orthotopic patient-derived tumours for panitumumab F(ab′)₂ fragments linked to MCPs with 2 pendant PEG₂K chains and 8 pendant PEG₂K chains was 7.31 ± 0.23 %ID/g and 4.9 ± 0.5 %ID/g, respectively. Orthotopic pancreatic patient-derived tumours were visualized by microPET/CT imaging for both of these RICs. These RICs showed low levels of liver and spleen uptake along with even lower levels of kidney uptake. These encouraging results confirm the stealth properties of the MCPs with pendant PEG₂K chains with prolonged retention in blood being achieved with increasing numbers of PEG₂K pendant chains.

Chapter 4 examined RIT *in vivo* with ⁶⁴Cu-panitumumab F(ab′)₂ fragments alone or combined with radiosensitizing GEM and the PARPi, rucaparib, in patient-derived pancreatic cancer tumour xenografts or PANC-1 pancreatic cancer xenografts in NOD-scid mice. Normal tissue toxicity was assessed in NOD-scid mice administered i.v. with 10 µg ⁶⁴Cu-panitumumab F(ab′)₂ or ⁶⁴Cu-anti-mouse EGFR F(ab′)₂ fragments (1.85-12.85 MBq). Body weight was monitored and
blood was analyzed for complete blood cell counts and serum alanine aminotransaminase and creatinine. No major normal tissue toxicity was observed with 

\[ ^{64}\text{Cu-panitumumab F(ab')}_{2} \] or 

\[ ^{64}\text{Cu-anti-mouse EGFR F(ab')}_{2} \] fragments. Treatment with 

\[ ^{64}\text{Cu-anti-mouse EGFR F(ab')}_{2} \] fragments (12.85 MBq) decreased hemoglobin, red blood cell counts, and hematocrit. No change in body weight occurred with any treatment. RIT studies were performed in NOD-scid mice engrafted s.c. with patient-derived OCIP23 pancreatic tumours treated with 

\[ ^{64}\text{Cu-panitumumab F(ab')}_{2} \] fragments (3.7 MBq) and subsequent RIT studies were performed in PANC-1 xenograft mouse models with 

\[ ^{64}\text{Cu-panitumumab F(ab')}_{2} \] fragments (12.95 MBq) alone or in combination with GEM and/or PARPi. OCIP23 patient-derived xenograft mice showed a slight tumour growth delay when treated with 3.7 MBq 

\[ ^{64}\text{Cu-panitumumab F(ab')}_{2} \]. PANC-1 xenograft mice exhibited no significant differences in median survival between control groups: normal saline, 32.2 ± 6.9 days; GEM + PARPi, 43.5 ± 9.7 days; 

\[ ^{64}\text{Cu-panitumumab F(ab')}_{2} \] fragments, 26.8 ± 14.3 days; and 

\[ ^{64}\text{Cu-panitumumab F(ab')}_{2} + \text{GEM} \], 54.7 ± 20.2 days. Mice treated with 

\[ ^{64}\text{Cu-panitumumab F(ab')}_{2} + \text{GEM} + \text{PARPi} \] survived longer than all control groups, 69.4 ± 11.7 days, except for 

\[ ^{64}\text{Cu-panitumumab F(ab')}_{2} + \text{GEM} \]. Mice treated with 

\[ ^{64}\text{Cu-panitumumab F(ab')}_{2} + \text{GEM} + \text{PARPi} \] had the greatest tumour growth delay. This chapter demonstrated that RIT with 

\[ ^{64}\text{Cu-panitumumab F(ab')}_{2} \] fragments delayed tumour growth and prolonged survival in pancreatic cancer mouse models when combined with GEM and PARPi. Also, evaluation of the normal tissue toxicity of the 

\[ ^{64}\text{Cu-anti-mouse EGFR F(ab')}_{2} \] fragments showed that there may not be significant EGFR-mediated toxicity from 

\[ ^{64}\text{Cu-panitumumab F(ab')}_{2} \] fragments in humans.

Chapter 5 aimed to determine the RBE of 

\[ ^{64}\text{Cu-} \text{and} \ ^{177}\text{Lu-panitumumab F(ab')}_{2} \] fragments in vitro by comparison to XRT γ-IR in 4 pancreatic cancer cell lines with varying degrees of EGFR expression, proliferation rates, and radiosensitivity, and one EGFR knockdown pancreatic cancer cell line. By determining the RBE, the studies in Chapter 5 aimed to investigate the response of these cell lines to 

\[ ^{64}\text{Cu-RIT,} \ ^{177}\text{Lu-RIT,} \] and XRT with γ-IR by clonogenic survival assays, and γ-H2AX analysis probing for DNA double-strand breaks based on equivalent radiation doses estimated by cellular dosimetry. Notably, the β-emitter 

\[ ^{67}\text{Cu}, \] may have been a more appropriate choice as a comparison to 

\[ ^{64}\text{Cu,} \] due to being the same element as well as having a shorter half-life than 

\[ ^{177}\text{Lu,} \] however, the high cost of this radionuclide prevented its use in these studies. This chapter further aimed to correlate the RBE with EGFR density and cell doubling times. To study the RBE of 

\[ ^{64}\text{Cu-} \text{and} \ ^{177}\text{Lu-labeled panitumumab F(ab')}_{2} \] fragments in comparison to XRT γ-IR
in 4 different pancreatic cancer cell lines EGFR density was measured on AsPC-1, PANC-1, MIAPaCa-2, and Capan-1 pancreatic cancer cells by flow cytometry. Relative EGFR density was high for AsPC-1 and PANC-1, low for MIAPaCa-2, and moderate for Capan-1 cells. Cell doubling time was determined over a 7 days period and found to be 60 h, 33 h, 26 h, and 71 h for AsPC-1, PANC-1, MIAPaca-2, and Capan-1 cells, respectively. Subcellular fractionation studies were performed in cells treated with $^{64}$Cu- or $^{177}$Lu-panitumumab F(ab')$_2$ fragments (1 MBq/72 nM) for 1, 3, 4, and 24 h. Radioactivity associated with cell surface, cytoplasm, and nucleus source compartments was used to estimate radiation doses to the cell nucleus. Cellular dosimetry was performed based on MCNP modeling to allow amounts of radioactivity incubated with pancreatic cancer cells (MBq) to be converted to absorbed dose (Gy) in clonogenic survival assays. Clonogenic survival assays were performed in all 4 cell lines where cells were treated with XRT $\gamma$-IR (0-8 Gy), $^{64}$Cu- or $^{177}$Lu-RIT (0-8 MBq/72nM) then seeded in into 6-well plates and allowed to grow at 37°C, 5% CO$_2$, for 14 days. Then colonies were fixed/stained and counted. The RBE was calculated using the dose required to reduce clonogenic survival to 10% ($D_{10}$) as the measurable effect. The $D_{10}$ values from clonogenic survival assays after $\gamma$-radiation for AsPC-1, PANC-1, MIAPaca-2, and Capan-1 cells were 3.2 Gy, 5.0 Gy, 3.7 Gy, and 2.5 Gy, respectively. The $D_{10}$ for $^{64}$Cu-RIT was 130 Gy, 13 Gy, 17 Gy, and 192 Gy for AsPC-1, PANC-1, MIAPaCa-2, and Capan-1 cells, respectively. The $D_{10}$ for $^{177}$Lu-RIT was 21 Gy, 26 Gy, 28 Gy, and 36 Gy, for AsPC-1, PANC-1, MIAPaCa-2, and Capan-1 cells, respectively. The associated RBE compared to XRT $\gamma$-IR for $^{177}$Lu-RIT was 0.15, 0.19, 0.13, and 0.07; and for $^{64}$Cu-RIT the RBE was 0.03, 0.38, 0.22, and 0.01, for AsPC-1, PANC-1, MIAPaCa-2, and Capan-1 cells, respectively. Chapter 5 demonstrated that the RBE did not correlate with EGFR density for either $^{64}$Cu- or $^{177}$Lu-RIT and that the RBE was not dependent on cell doubling time for $^{177}$Lu-RIT, while for $^{64}$Cu it was dependent on cell doubling time with a greater RBE found for fast proliferating cell lines, PANC-1 and MIAPaCa-2. Additionally, Chapter 5 showed that a higher radiation dose is required for RIT with $^{64}$Cu- or $^{177}$Lu-labeled panitumumab F(ab')$_2$ fragments than XRT $\gamma$-IR to reduce the clonogenic survival of pancreatic cancer cell lines to 10%. Finally, Chapter 5 demonstrated that the trend in radioresponsiveness between pancreatic cancer cell lines is not maintained when comparing XRT $\gamma$-IR and RIT with $^{64}$Cu or $^{177}$Lu-panitumumab F(ab')$_2$ fragments, where PANC-1 was most radioresistant to treatment with XRT and least radioresistant to $^{64}$Cu-RIT, while little variation in response was observed between cell
lines following treatment with $^{177}$Lu-RIT, most likely due to the slower dose rate which gives cells an opportunity to repair DNA damage prior to mitosis.

### 6.2 Future directions

The Chapters of this thesis provide interesting and enlightening information about the use of $^{64}$Cu as a theranostic agent, enhancing $^{64}$Cu-RIT with radiosensitizers, as well as providing a comparison of $^{64}$Cu and $^{177}$Lu as radionuclides for RIT of pancreatic cancer. The findings of these chapters give rise to insight into new avenues of investigation as well as practical future research that would provide further information on the utility of panitumumab as the targeting moiety for anti-EGFR theranostics for imaging and RIT of pancreatic cancer.

Chapter 2 provided the first step in developing a theranostic agent for pancreatic cancer by demonstrating the potential of $^{64}$Cu-panitumumab F(ab')$_2$ fragments for PET/CT imaging of orthotopic pancreatic patient-derived xenografts. Chapter 4 described the next step in assessing $^{64}$Cu-panitumumab F(ab')$_2$ fragments as a theranostic agent by investigating the utility of this agent in RIT studies *in vivo* in combination with radiosensitizing agents. In this study, I was able to deliver as much as a total of 78 MBq in 12.95 MBq fractionated doses when combined with GEM and PARPi, with no observable signs of toxicity. However, neither my studies nor those described previously in the literature with $^{64}$Cu-labeled mAbs demonstrate tumour growth arrest [57, 58], leading me to the conclusion that other β-particle emitting radionuclides may be more appropriate for future studies if regression of a solid tumour is the desired primary outcome.

This, in turn, paved the way for Chapter 5, in which I investigated the differences in response of pancreatic cancer cells *in vitro* to RIT with $^{64}$Cu and $^{177}$Lu-panitumumab F(ab')$_2$ fragments. In general, when pancreatic cancer cells were treated with $^{177}$Lu-RIT their responses were similar and, in most cases, better than when treated with $^{64}$Cu-RIT. Of particular importance, this study showed that the slow growing, metastatic cell lines, AsPC-1 and Capan-2, were the least responsive to $^{64}$Cu-RIT. Since metastatic disease is perhaps the best target for RIT, I would conclude that future studies should move forward with $^{177}$Lu as the radionuclide for RIT. $^{177}$Lu can be applied as a theranostic agent since it has low abundance γ-emissions that can be used for SPECT imaging. Based on the evidence provided in Chapter 5 combined with the goal of developing a single theranostic agent for both imaging and therapy of pancreatic cancer, a practical future investigation should look into the effectiveness of SPECT imaging using $^{177}$Lu.
for visualizing orthotopic patient-derived pancreatic cancer tumours in mice. Additionally, in vivo therapy studies should be performed similar to those described in Chapter 4. If SPECT imaging is successful with $^{177}$Lu-panitumumab F(ab′)$_2$ fragments and tumour regression is observed with in vivo RIT studies, this agent would prove to be an excellent candidate to move forward for in-human clinical studies. Prior to moving into these studies, a thorough stability study should be performed comparing NOTA and DOTA as chelators for $^{177}$Lu since DOTA is well established as a strong chelator for $^{177}$Lu, and although the stability of $^{177}$Lu-NOTA has been demonstrated previously in the literature [55, 246], these studies were not performed in mouse serum, but rather in PBS, and so the relevant in vivo stability of $^{177}$Lu-NOTA has not been demonstrated in the literature.

A very exciting future study should examine the in vitro and in vivo efficacy of an α-emitter conjugated to panitumumab for RIT. An α-emitter, such as $^{225}$Ac, could deposit more ionizing radiation in cancer cells while causing less normal tissue toxicity. Although this radionuclide could not be employed as a single theranostic agent for both diagnosis and treatment of pancreatic cancer because there are no gamma emissions from $^{225}$Ac for imaging, the potential of an α-emitter for robust RIT should not be overlooked. I recommend studying $^{225}$Ac since its 10 day half-life is comparable to that of $^{177}$Lu which has a 6.7 day half-life. I would suspect the efficacy in vitro to be greater for the α-emitter, $^{225}$Ac, than for the β-emitter, $^{177}$Lu, since RBE studies comparing α-emitters as RIT have reported RBEs greater than 1, while $^{177}$Lu-RIT is often reported as being less than 1 [239, 249]. However, despite having a higher RBE, a β-emitter may be have advantages in vivo. For example, in a study comparing high-LET $^{227}$Th-trastuzumab, an α-emitter, and low-LET $^{177}$Lu-trastuzumab targeting HER2 in mice bearing SKBR-3 human breast cancer xenografts, the authors found the RBE to be higher for $^{227}$Th-trastuzumab when these RICs were compared at the same therapeutic effect of 100% prolonged tumour growth delay compared to the control [66]. Yet the therapeutic index was greater for $^{177}$Lu-trastuzumab as determined when the RICs were compared at the same normal tissue toxicity, i.e. decrease in white blood cell count of 50% compared to control, for which $^{177}$Lu-trastuzumab had 3 times longer tumour growth delay [66]. It is important to perform RBE studies in tumour bearing mice to ascertain an RBE that reflects an in vivo environment where both the effects on the tumour as well as normal tissues are considered. A possible explanation of the therapeutic window being greater for $^{177}$Lu compared to $^{227}$Th in RIT studies lies in the issue of recoil energy being
released during $\alpha$-particle decay combined with conversion of the parent radionuclide to a daughter radionuclide with different chelation chemistry which can lead to the release of radioactivity from the targeting moiety, as depicted in Figure 6.1, which may redistribute to normal tissues, potentially causing toxicity. The recoil energy resulting from $^{225}$Ac may not be as detrimental as that observed in the study referenced above with $^{227}$Th, and so, the therapeutic window may be comparable to $^{177}$Lu and allow the greater RBE to ultimately provide greater tumour regression.

The final area for future investigation involves the fact that studying panitumumab in mouse models raises a point of concern because panitumumab does not bind to murine EGFR. Although I show in Chapter 4 that $^{64}$Cu-labeled anti-mouse EGFR does not cause significant toxicity to normal mouse tissues, this is only a speculative indication of the effect anti-EGFR RIT may have in humans with panitumumab as the targeting moiety. Radiolabeled panitumumab has been studied as a PET imaging agent in patients with metastatic colon cancer who were injected with 37 MBq of $^{89}$Zr-panitumumab where no significant toxicity was observed [116], however, the half-life of $^{89}$Zr is much shorter than both $^{177}$Lu and $^{225}$Ac, and so, the toxicity profile may be very different for these radionuclides. Additionally, if the results of a future in vivo RIT study comparing $^{177}$Lu and $^{225}$Ac are performed, as recommended above, the recoil energy caused from $\alpha$-decay and the subsequent conversion of the parent radionuclide to daughter a radionuclide with different chelation chemistry may lead to the release of the $\alpha$-emitter from the targeting moiety resulting in increased normal tissue toxicity. Performing toxicity studies in non-human primates would provide relevant information about the normal tissue toxicity of radiolabeled panitumumab since cynomolgus monkey EGFR cross-reacts with panitumumab [228]. I recommend performing toxicity studies in cynomolgus monkeys with either $^{177}$Lu or $^{225}$Ac-labeled panitumumab F(ab')$_2$ fragments, depending on the results of the RIT studies recommended above.
Figure 6.1. The recoil energy released during α-particle decay combined with conversion of the parent radionuclide to a daughter radionuclide with different chelation chemistry results in release of radioactivity from the monoclonal antibody which may redistribute to normal tissues, potentially causing toxicity. The example shown is for a monoclonal antibody labeled with $^{225}\text{Ac}$ which decays to $^{221}\text{Fr}$ by release of an α-particle. Reprinted with permission from Aghevlian S [44].
References


