The Synthesis and Evaluation of Peptide-Based Probes for the Imaging of RHAMM Expressing Carcinoma and GLP-1R on Pancreatic Beta Cells

Ashley Esarik

Supervisor
Dr. Leonard Luyt

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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The Synthesis and Evaluation of Peptide-Based Probes for the Imaging of RHAMM Expressing Carcinoma and GLP-1R on Pancreatic Beta Cells

(Thesis format: Monograph)

by

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Graduate Program in Chemistry
In Collaboration with Molecular Imaging

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

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THE UNIVERSITY OF WESTERN ONTARIO
Abstract

Peptide-based probes are developed into imaging agents to target two different receptors: receptor for HA mediated motility (RHAMM) and glucagon-like peptide 1 receptor (GLP-1R).

In the first project, peptides are derived from tubulin and mimic HA (hyaluronan), the natural ligand of RHAMM. In order to develop the lead candidate peptides into imaging agents, a DOTA chelator which can coordinate a radiometal was coupled onto the lead peptides. Gallium labelling studies were performed and the labelled probes were used in *in vivo* mouse studies. Various analogues of two of the candidate peptides were also synthesized using bioisosteric, unnatural as well as natural amino acid replacements. They were incubated with RHAMM-coated magnetic/fluorescent beads and screened using a plate reader. This project resulted in the conclusion that the peptides containing only natural amino acids are not adequate imaging agents, but led to the discovery of novel peptides that have more potential as probes targeting RHAMM *in vivo*.

In the second project, truncated GLP-1 analogues were synthesized and characterized. The unnatural amino acids, BIP 1 and BIP 2 were first synthesized before being incorporated into the peptides. The potential of the peptides were analyzed by binding assays which determine the IC$_{50}$ and EC$_{50}$. The two DOTA chelated peptides were coordinated with $^{68}$Ga and *in vitro* cell studies were performed. Ultimately, this research found an allosteric agonist for the GLP-1R which may have the potential to be a PET imaging probe to monitor type 2 diabetes.

**Keywords**

Molecular imaging, radiochemistry, gallium-68, hyaluronan, receptor for hyaluronan mediated motility (RHAMM), tubulin, carboxy terminal tail, bioisostere, breast cancer, diabetes, GLP-1R
Co-Authorship Statement

Chapter 2: this work was completed in collaboration with the laboratory of Dr. Eva Turley at the London Regional Cancer Program. ELISA assays from the alanine scans were completed by Natalia Akentieva, and the ELISA assay for RHAMM and tumour mouse injections were completed by Dr. Cornelia Toelg. Fluorescence microscopy and cell culture was carried out by Dr. Patrick Telmer. Mouse injections with the probe and biodistribution studies were completed by Dr. Lihai Yu.

Chapter 3: Cell culture as well as binding assays (IC$_{50}$ and EC$_{50}$) were carried out by Rebecca McGirr.

All other work in the chapters was completed by the author. This includes all synthesis, purification, characterization, labelling, $^{68}$Ga in vitro binding affinity assays, bead incubation and screening.
Acknowledgements

First, I’d like to thank my supervisor, Dr. Len Luyt. You have always been available for advice and to help guide me in the right direction while still inspiring freedom of thought. Thanks for the opportunity to work in your lab; I have learned a lot in the brief time.

Next, I’d like to thank the Turley and Dhanvantari lab for their help with the biological studies. Specifically, Natalia, Pat and Conny (Turley) and Rebecca (Dhanvantari). I would not have been able to complete either project without your assistance. I also thank Dr. Mike Kovacs for the use of his radiochemistry facilities and the Deroo lab for the use of their plate reader.

I’d also like to thank the members of the Luyt lab, past and present. Your knowledge and assistance have really helped me grow as a chemist. A special thanks to Dr. Lihai Yu for his help with mice injections and biodistribution studies and our lab technician, André St. Amant, who was always there when I had questions or to fix equipment.

I thank the financial support from the Translational Breast Cancer Research Unit at the London Regional Cancer Program.

Last, but certainly not least are my friends and family. Old and new friends alike, you guys have been amazing friends and we have shared some of the best times. Even when you weren’t aware, many of you helped me get through some tough times and endure through the many hurdles during my graduate studies. The most thanks are owed to my family: my mom, dad and my brother, Shane. I want to thank you all for your unconditional love and support. Thanks for suffering through my many chemistry rants about my research, even if you didn’t understand much of what I was saying. If it wasn’t for all your sacrifices, I would not be where I am today.
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEEA</td>
<td>2-[2-(2-aminoethoxy)ethoxy]acetic acid</td>
</tr>
<tr>
<td>Ahx</td>
<td>aminohexanoic acid</td>
</tr>
<tr>
<td>Aib</td>
<td>α-aminoisobutyric acid</td>
</tr>
<tr>
<td>BIP</td>
<td>biphenylalanine</td>
</tr>
<tr>
<td>Boc</td>
<td>t-butoxycarbonyl</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CHO</td>
<td>chinese hamster ovary</td>
</tr>
<tr>
<td>CTT</td>
<td>carboxy terminal tail</td>
</tr>
<tr>
<td>DOTA</td>
<td>1,4,7,10-triazacyclododecane-1,4,7,10-tetraacetic acid</td>
</tr>
<tr>
<td>DPP-IV</td>
<td>dipeptidyl-peptidase-IV</td>
</tr>
<tr>
<td>EI</td>
<td>electron impact</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>FDG</td>
<td>2-deoxy-2-[18F]fluoro-D-glucose</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Fmoc</td>
<td>9-fluorenylethoxycarbonyl</td>
</tr>
<tr>
<td>GBq</td>
<td>gigabecquerel</td>
</tr>
<tr>
<td>GLP-1R</td>
<td>glucagon-like peptide-1 receptor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronan</td>
</tr>
<tr>
<td>HARE</td>
<td>hyaluronan receptor for endocytosis</td>
</tr>
<tr>
<td>HATU</td>
<td>2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium</td>
</tr>
<tr>
<td>HCTU</td>
<td>O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>K0</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>keV</td>
<td>kiloelectron volts</td>
</tr>
<tr>
<td>MAPs</td>
<td>microtubule associated proteins</td>
</tr>
<tr>
<td>NBS</td>
<td>N-bromosuccinimide</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>RHAMM</td>
<td>receptor for hyaluronan mediated motility</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>reverse-phase high performance liquid chromatography</td>
</tr>
<tr>
<td>SPECT</td>
<td>single-photon emission computed tomography</td>
</tr>
<tr>
<td>SPPS</td>
<td>solid-phase peptide synthesis</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>TIPS</td>
<td>trisopropylsilane</td>
</tr>
<tr>
<td>UHPLC</td>
<td>ultra high performance liquid chromatography</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Molecular Imaging

Imaging techniques have helped shape our understanding of various diseases. Recently, it has become possible to visualize specific molecules and targets. This research discipline is called molecular imaging and is defined as a method to non-invasively visualize, characterize and quantify biological processes at the molecular and cellular levels. Molecular imaging has been shown to play a significant role in modern medicine due to its potential application in diagnostics and therapeutics. In order for the non-invasive in vivo visualization of biological processes to be feasible, it requires the development of an appropriately designed imaging probe.

1.2 Imaging Probes

The non-invasive visualization of biological processes in vivo requires the discovery and development of novel imaging agents. There are two main classes of probes: non-specific and targeted. Non-specific probes, instead of interacting with specific receptors, emphasize dissimilarities between tissues relating to permeability and perfusion levels and have compartmental distributions. The design of non-specific agents relies on general chemical properties such as size, charge and hydrophilicity to determine the distribution in the body. These probes are typically used to image physiological processes such as changes in blood volume, perfusion and blood flow in angiogenesis. Often the compartmental distribution changes over time, so fast imaging might be required. There are numerous non-targeted probes that currently exist in the clinic, such as PET (¹³N-ammonia) and SPECT (⁹⁹mTc-sestamibi) probes used for perfusion imaging. Also, gold nanoparticles that target tumours via the enhanced permeability and retention effect are being utilized in pre-clinical studies.
In contrast to non-specific probes, targeted probes, the strictest category of molecular imaging, are designed to target a specific site within the body such as a breast cancer tumour. The probe typically consists of two main components: the targeting entity, which is a chemical that can be a small molecule, peptide or other biomolecule able to recognize and bind with high affinity to a particular biological receptor, and the label which provides the signal for detection (Figure 1.2).

![Figure 1.1 Structure of $^{99m}$Tc-sestamibi$^6$](image)

Figure 1.1 Structure of $^{99m}$Tc-sestamibi$^6$

Depending on the modality of choice, the label can be organic fluorophores or inorganic quantum dots for optical imaging$^8$ or radioisotopes for nuclear imaging. Sometimes, there may be a linker present to add distance between the label and the targeting entity to allow for proper receptor-ligand binding. Targeted probes are detectable at all times, regardless of any interaction with the biological target, which
leads to higher background noise levels. However, considering that only the probes bound to the biological targets stay in the system, higher signal to noise ratios can be obtained if waiting periods are permitted prior to imaging. There are a number of necessary prerequisites for a targeting entity to be useful in nuclear medicine. The targeting entity must preferentially go to a desired part of the body and be able to move quickly to the area of interest, because of the time constraints imposed by using radionuclides. Additionally, the targeting moiety should not produce an immune response within the patient as well as being able to be cleared from the body after the imaging has been completed in order to minimize exposure to radiation. As a result of the conditions necessary for a useful targeting entity, the development of molecular imaging agents relies heavily on the discovery and development of new targeting entities.

### 1.3 Peptides as Targeting Entities

As discussed, the targeting component can range between being a small molecule, peptide or antibody. Small molecules have several attractive qualities as targeting entities. They often have low molecular weight which allows access to many biological targets in the body. Small molecules are not easily detected and degraded by enzymes. They can be modified to produce many diverse compounds, but the synthesis may be difficult and time consuming and rarely leads to suitable imaging agents. Another important shortcoming is the limiting number of radionuclides that can be incorporated into small molecules. Due to their low molecular weight, a bulky metal radionuclide, such as $^{68}$Ga, will often drastically lower the affinity. In this report, peptide-based compounds are used as the targeting component for the development of molecular imaging probes. Peptides have many advantages over other targeting entities.
Synthesis of peptides using automated solid-phase methods allow for easy and quick production of analogues to improve their stability and increase their half-life in biological systems.\textsuperscript{11} Moreover, peptides are able to withstand harsh chemical conditions such as radiolabelling conditions often requiring elevated temperatures and organic solvents.\textsuperscript{12} Due to their higher molecular weight compared with small molecules, they are able to incorporate a large variety of radionuclides including the bulky metal radionuclides, without considerably affecting their binding affinity. Peptides have also been shown to possess high affinity for their target at nanomolar concentrations. In comparison to macromolecules, peptides have high tissue and tumour permeability and faster clearance rates due to their low molecular weights.\textsuperscript{11, 13} In addition, there are an increasing number of unnatural amino acids that are commercially available, thereby extending the chemical diversity of peptides. Due to the many benefits peptides provide over other targeting molecules, they are becoming more widespread in their use as imaging agents. This can be seen in the increasing number of peptide-based imaging agents in clinical trials. Some examples include \[^{18}\text{F}]\text{Galacto-RGD, which is used to monitor tumour growth and metastasis}^\text{(Figure 1.3)}\) and \[^{99}\text{mTc-UBI 29-41 (ubiquicidin), used to image infection.}^\text{15}\)

\begin{center}
\includegraphics[width=0.5\textwidth]{Figure1.3.png}
\end{center}

\textit{Figure 1.3} \[^{18}\text{F}]\text{Galacto-RGD, a PET imaging agent for oncologic applications}\)
The design of a suitable probe needs to account for biological stability, target affinity, target specificity and pharmacokinetic properties. One must design, synthesize and evaluate the lead peptides in vitro, and determine if further optimization is required. Biological evaluation may consist of competitive binding assays and serum stability studies. The peptides showing the best affinity and specificity to the target would move on to in vivo studies in small animal models.

1.4 Radionuclide Labeling

Common labels used in molecular imaging are fluorescent dyes, radioisotopes, and paramagnetic agents, of which radioisotopes are used in nuclear imaging. The most common method to incorporate radionuclides is an indirect approach, where a radiometal chelator is conjugated onto the biological compound of interest and labelled with the radionuclide. Bifunctional metal chelators have recently gained much interest due to their high radiolabelling yields and good stability.\textsuperscript{16} The term bifunctional is used because they have a metal binding moiety function and also possess a chemically reactive functional group for covalent attachment to the targeting entity. The radiolabelling of the chelator can be carried out using either pre-labeling or post-labeling methodologies. In the first method, the radiolabeling is carried out initially, and afterwards the isotope-chelator complex is conjugated to the targeting entity. However, this method is not ideal because the purifications steps are time consuming and the radioisotopes used are short-lived.\textsuperscript{17} More commonly, the post-labeling approach is used in which the chelator is already conjugated to the peptide entity and the complex is radiolabeled. This method is more suited for the time sensitive radionuclide and often results in high yields.\textsuperscript{16} There are a variety of bifunctional chelators that are available to coordinate radiometals. These include DOTA, NOTA and DTPA (Figure 1.4).\textsuperscript{18-21}
Figure 1.4 Examples of common bifunctional chelators that coordinate radiometals. A. DOTA  B. NOTA  C. DTPA

Linear polyamines such as DTPA (diethylenetriaminepentaacetic acid) can act as both a monofunctional and bifunctional chelator and has shown to coordinate a variety of radionuclides, such as $^{111}$In. The disadvantage of these anionic acyclic systems is that they tend to demonstrate low stability in vivo because of acid-promoted dissociation. A more stable alternative are macrocyclic bifunctional chelators such as DOTA (1,4,7,10-triazacyclododecane-1,4,7,10-tetraacetic acid) and NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid). They have been shown to form stable complexes with trivalent metals such as Ga, In, Y and Lu as well as some divalent metals. The high stability is usually associated with the slow dissociation reactions observed for the neutral complexes. Ideally, the chelator that is conjugated to a peptide should coordinate the radionuclide quickly and satisfactorily. Next, the complex should be stable to a pH range of 2-8 and in the presence of various cations found in serum [Ca(II), Zn(II)]. Finally, factors such as charge and lipophilicity should be considered. The choice of radionuclide is also important, and factors such as the half-life, availability and mode of decay have to be considered. The half-life should be long enough for the injection of the probe, its accumulation in the target tissues and ultimately its clearance and yet short enough to limit exposure to radioactivity.
1.5 Imaging Modalities

Two of the most common modalities in nuclear imaging are single-photon emission computed tomography (SPECT) and positron emission tomography (PET). Both modalities require only a small amount of the radioactive tracer which is then injected into the body to non-invasively monitor the target of interest. Currently, PET offers higher resolution and sensitivity whereas SPECT offers longer lived radioisotopes and lower costs.23 However, with advances in hardware and software, SPECT imaging is greatly improving and even surpassing small animal PET systems in spatial resolution.24,25 PET uses positron emitters and SPECT uses gamma emitters, though in both modalities gamma rays are detected.24 Some examples of common radionuclides used in PET and SPECT are listed in Table 1.1.

Table 1.1 Common radioisotopes used in PET/SPECT imaging, along with their respective half-life and method of synthesis26

<table>
<thead>
<tr>
<th>Radioisotope</th>
<th>Half-life</th>
<th>Modality</th>
<th>Method of Synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen-15</td>
<td>2.03 min</td>
<td>PET</td>
<td>cyclotron</td>
</tr>
<tr>
<td>Carbon-11</td>
<td>20.3 min</td>
<td>PET</td>
<td>cyclotron</td>
</tr>
<tr>
<td>Gallium-68</td>
<td>67.7 min</td>
<td>PET</td>
<td>generator</td>
</tr>
<tr>
<td>Fluorine-18</td>
<td>109.8 min</td>
<td>PET</td>
<td>cyclotron</td>
</tr>
<tr>
<td>Technetium-99m</td>
<td>6.02 hours</td>
<td>SPECT</td>
<td>generator</td>
</tr>
<tr>
<td>Iodine-123</td>
<td>13.2 hours</td>
<td>SPECT</td>
<td>cyclotron</td>
</tr>
<tr>
<td>Indium-111</td>
<td>2.83 days</td>
<td>SPECT</td>
<td>cyclotron</td>
</tr>
<tr>
<td>Gallium-67</td>
<td>3.26 days</td>
<td>SPECT</td>
<td>cyclotron</td>
</tr>
</tbody>
</table>

In PET, a positron-emitting isotope is introduced into the body conjugated to a biologically active molecule. The radioisotope emits a positron from its nucleus, which travels a short distance before it annihilates with a nearby electron (Figure 1.5).11 Upon annihilation, the mass of the proton and electron are converted into energy producing two simultaneous 511 keV gamma rays 180° from each other.26 These gamma rays are
detected by a set of adjacent detectors and the information gathered is used to construct an image.

![Diagram](image)

**Figure 1.5** Method of detection in PET

In PET, there are three types of coincident events: random, scatter and true coincidences. Random coincidence occurs when photons from different nuclear decays are detected nearly simultaneously and are a source of undesirable background counts. In scattered coincidence, one or more photons change direction from a scatter before detection. Finally, true coincident events are the ones desired; anti parallel photons travel directly to the detectors. The distance travelled by the positron is known as the positron range and is proportional to the positron energy. It is one of the factors that affect the spatial resolution in PET. Other factors include patient movement, detector size and photon non-collinearity. Since radionuclides produce distinct positron energies, they also have varying flight distances, which in turn affect the spatial resolution in the image. This is especially significant in certain tissues, such as lung tissue, in which positron contribution was found to be three fold higher than in soft tissue. PET is very sensitive in the range of pM and is independent of the location depth of the tracer. There are a number of important implications that result from the high sensitivity, such as improved image quality, shorter and multiple scans and
improved temporal resolution. Additionally, PET radiotracers generally have shorter half-lives, so can be injected in higher activities to patients without causing greater radiation exposure in comparison to SPECT. All PET isotopes produce gamma rays of the same energy, consequently it is impossible to distinguish multiple probes containing different radioisotopes. There are many PET radionuclides used, such as $^{18}$F, $^{15}$O and $^{11}$C, and $^{68}$Ga. As a result of the versatility of PET, there are a variety of PET radiotracers and clinical applications such as $^{18}$F-FDG, which is considered the golden standard in PET imaging and used in radiotherapy planning in various cancers such as neck, lung and head.

SPECT is similar to PET, however, they differ in the type of radioisotope used. In SPECT, the gamma detector rotates around the patient and the gamma ray radiation is detected directly from the radionuclide. Directional information is obtained from the use of a collimator in front of the gamma camera which defines the angle of incidence of the emitted gamma rays. The loss of sensitivity and resolution compared with PET is caused by the collimator which results in low detection efficiency. Advances in the design of collimators such as multi-hole collimators have greatly improved the resolution and sensitivity of SPECT. Whereas the spatial resolution of PET is limited by physics, SPECT is only limited by technology and is constantly being enhanced. One important advantage of SPECT is that it allows for the simultaneous detection of multiple isotopes with varying gamma ray energies, such as in the study by Hashimoto et al using both $^{99m}$Tc and $^{123}$I to image myocardial perfusion. This could potentially visualize multiple probes targeting distinct markers, and ultimately a single image of a cancer’s molecular expression profile. The most common radioisotopes used are $^{111}$In and $^{99m}$Tc. $^{99m}$Tc has a half-life of 6 h which allows for the preparation, distribution to hospitals, accumulation in the target tissue, and collection of the image.
Radioactive decay can occur by a number of mechanisms such as electron capture in which a proton in the nucleus combines with an electron to form a neutron and a gamma ray. However, radionuclides such as technetium-99m undergo gamma emission whereby a nucleus in a high energy state relaxes over time via the emission of a gamma ray. The electromagnetic radiation is then detected by a gamma camera and an image is formed.

The use of either PET or SPECT is greatly dependent on the radionuclide and therefore the suitability of the probe and availability of the radionuclide and imaging equipment.

### 1.6 Radionuclide Production

Most short-lived radionuclides are neutron deficient and therefore can only be prepared in positive ion accelerators. In particular, cyclotrons are currently the preferred type of particle accelerator and are becoming much more common than in the past. The first cyclotron was built in 1930 with proof of particle acceleration provided in 1931. The bombarding particles are typically protons, but can also be deuterons or helium particles. There are a large number of nuclear reactions that can be performed to produce various radioisotopes, including $^{18}\text{F}$, $^{111}\text{In}$ and $^{68}\text{Ge}$.

#### Table 1.2 Common radionuclides produced in a cyclotron and their nuclear reactions

<table>
<thead>
<tr>
<th>Radioisotope</th>
<th>Half-life</th>
<th>Nuclear Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorine-18</td>
<td>109.8 min</td>
<td>$^{18}\text{O}(p,n)^{18}\text{F}$</td>
</tr>
<tr>
<td>Indium-111</td>
<td>2.8 days</td>
<td>$^{111}\text{Cd}(p,n)^{111}\text{In}$</td>
</tr>
<tr>
<td>Germanium-68</td>
<td>272 days</td>
<td>$^{68}\text{Ga}(p,2n)^{66}\text{Ge}$</td>
</tr>
</tbody>
</table>

The interest in quick and convenient radioisotope production lead to the development of generators. Generators are simple and convenient to use and provide...
high yields of the daughter nuclide reproducibly on demand. They are shielded thus minimizing radiation exposure and their shipment is simple since they are quite sturdy. Generators are used for effective radiochemical separation of a daughter radionuclide formed by the decay of a parent radionuclide. The parent typically has a half-life much longer than the daughter to permit repeated separation of the daughter from the parent. Differences in the chemical properties of the two allow for the efficient isolation of the daughter radionuclide. There exist generator systems producing either positron emitters ($^{68}$Ge/$^{68}$Ga) or gamma emitters ($^{99m}$Mo/$^{99m}$Tc). In general, the parent radionuclide is adsorbed onto the column packing and decays into the daughter until an equilibrium is reached. This occurs within several half-lives of the daughter at which point the half-life of the daughter would appear to be the same as that of the parent. When the generator is eluted, the daughter would be eluted from the column, leaving the parent still adsorbed to the column because of their differing chemical properties.

**1.7 $^{68}$Ge/$^{68}$Ga Generator**

One very important generator system is the $^{68}$Ge/$^{68}$Ga generator. The relatively long-lived $^{68}$Ge (half-life = 272 days) decays to short-lived $^{68}$Ga (half-life = 68 min), which ultimately decays to stable $^{68}$Zn. The chemical properties of Ge$^{4+}$ and Ga$^{3+}$ are sufficiently different to allow several methods for efficient separation. There are two main strategies used: organic matrices and inorganic oxides as matrices. One study used an organic polymer based on N-methylglucamine groups which was shown to rapidly and effectively adsorb $^{68}$Ge. After elution with trisodium citrate, the elution efficiency of $^{68}$Ga was 90% with $^{68}$Ge breakthrough less than 0.0004%. Unfortunately, organic polymers have low radiation stability compared to their inorganic competitors. The second strategy uses inorganic oxides such as Al$_2$O$_3$, SnO$_2$ or TiO$_2$. Though the alumina column
provides efficient separation, $^{68}$Ga is eluted as a $^{68}$Ga-EDTA complex. This leads to complicated and tedious synthesis of radiopharmaceuticals, thus generators providing $^{68}$Ga$^{3+}$ were developed. The SnO$_2$ and TiO$_2$ columns allow the elution of $^{68}$Ga$^{3+}$ using HCl. It has been shown that at a concentration of 0.1 M HCl, TiO$_2$ shows the highest affinity for Ge (>95% adsorption) and lowest affinity for Ga (~0%) compared with Al$_2$O$_3$ and SnO$_2$. Various methods have been developed to purify $^{68}$Ga$^{3+}$ from $^{68}$Ge including the use of anion and cation exchange columns as well as Sep-Pak purification. The current approaches use different $^{68}$Ga species depending on the pH and concentration of eluent. The most popular method uses a cation exchange column, eluting with a HCl/acetone solution. It is a very reliable method that produces the product in high radiochemical purity, but uses organic solvents which requires quality control testing of the final products. More recent work has focused on a NaCl-based labelling procedure for DOTA conjugated peptides. It has eliminated the need for organic solvents yet maintains very high radiochemical purities. It is evident that generators, specifically the $^{68}$Ge/$^{68}$Ga generator, have many advantages in producing radionuclides. Their shipment is easy, are convenient to use and provide high yields of $^{68}$Ga reproducibly in high radiochemical purity.

1.8 Chemistry of $^{68}$Ga

$^{68}$Ga has a convenient half-life of 68 minutes, which is long enough for experiments while minimizing the radiation exposure to the patient. It is an excellent positron emitter decaying 89% by positron emission and 11% by electron capture with a maximum positron energy of 1899 keV. The coordination chemistry of Ga$^{3+}$ is well established and accordingly the chelator is designed to avoid in vivo transchelation of $^{68}$Ga$^{3+}$. Radiochemical yields are determined by the ability of the chelator to coordinate
Ga\textsuperscript{3+} from highly diluted solutions.\textsuperscript{45} As previously mentioned, common chelators are DTPA, DOTA and NOTA. Macrocyclic chelators will demonstrate selectivity for metal ions based on how well the ion fits into the cavity.\textsuperscript{46} There are a wide array of applications that involve DOTA peptides including magnetic resonance imaging (Gd), nuclear imaging (\textsuperscript{111}In and \textsuperscript{68}Ga) and therapeutic radiopharmaceuticals (\textsuperscript{90}Y).\textsuperscript{47} Some examples of DOTA conjugated to peptides are comprised of DOTA labelled somatostatin analogues targeting neuroendocrine tumours (DOTA-octreotide),\textsuperscript{48, 49} GLP-1R agonists, (DOTA-exendin)\textsuperscript{50,51} and various RGD peptides involved in angiogenesis.\textsuperscript{52}

The complexes formed should have high thermodynamic stability. The stability of the complexes tend to follow the hard-soft acid-base theory. Gallium is classified as a hard Lewis acid because of its high charge density and small ionic radius and therefore forms stable complexes with ligands that are hard Lewis bases, such as oxygen and nitrogen atoms.\textsuperscript{53,54} Due to its small size (0.62 Å), it is often six-coordinate as a distorted octahedral. Gallium can also form four- and five-coordinate complexes, and be adequately stable in vivo. However, it is desirable to saturate the coordination sphere since unsaturated complexes often result in ligand exchange/hydrolysis.\textsuperscript{23} Under physiological conditions, the solution chemistry in aqueous media of gallium is solely represented by the oxidation state +3. An important consideration of the chemistry complexation of gallium is its pH dependent hydrolysis. Free Ga\textsuperscript{3+} is only stable under acidic conditions and can hydrolyze to insoluble trihydroxide [Ga(OH)\textsubscript{3}] in the pH range 3.5-7. At physiological pH, soluble gallate ions [Ga(OH)\textsubscript{4}]\textsuperscript{−} are formed.\textsuperscript{55} Hence, it is important for the optimization of pH levels and ligands for stable complexes to form during the reaction. Overall, the \textsuperscript{68}Ga coordinated macrocyclic chelators, such as DOTA, have shown high stability and biological inertness.\textsuperscript{55-57}
$^{68}$Ga is a PET radioisotope of great interest due to its convenient physical half-life, accessibility and cost. There have been increasing examples in the literature of $^{68}$Ga labelled imaging agents for clinical applications. For instance, it has been used to visualize tumours,\textsuperscript{58} bone disorders,\textsuperscript{59} infection and inflammation such as \textit{Staphylococcus aureus} infection and the detection of abscesses ($^{68}$Ga-citrate),\textsuperscript{60} diabetes, as well as many others.

### 1.9 Thesis Rationale

In this thesis we report on peptide-based probes that are developed into imaging agents to target two different receptors: receptor for HA mediated motility (RHAMM) and glucagon-like peptide 1 receptor (GLP-1R). In the first project, peptides derived from tubulin were synthesized and characterized in the hopes to mimic hyaluronan (HA), the natural ligand of RHAMM, a protein that is overexpressed in breast cancer tumours. In the past, HA has been labelled with radionuclides ($^{125}$I and $^{99m}$Tc) and MRI contrast agents (Gd$^{3+}$) for its use as an imaging probe.\textsuperscript{61} Unfortunately, this poses several problems; the most important being its potential to target any of its numerous receptors (CD44, ICAM1, RHAMM, HARE), resulting in its non-specificity. Peptides as imaging agents provide a more promising alternative. The main advantages are the ability for the peptides to be designed to specifically target RHAMM and to not be recognized for HA clearance or degradation. Peptide ligands that show affinity and specificity for RHAMM were discovered through bioinformatics, biological assays and \textit{in vitro} studies. In the second project, truncated analogues of GLP-1 will be synthesized and characterized, designed to target the glucagon-like peptide 1 receptor (GLP-1R) in the pancreas. The objective for both projects is to develop the lead peptides into imaging agents through the incorporation of a DOTA chelator and labelling with $^{68}$Ga. The imaging agents will be
evaluated via *in vitro* studies, followed by *in vivo* studies and biodistribution to assess the affinity of the probes to their respective receptors, RHAMM and GLP-1R. Ultimately, this dissertation aims to synthesize and characterize imaging agents that may potentially allow: 1) for the earlier, selective detection of primary and metastatic breast cancer as well as 2) the monitoring of pancreatic beta cell mass and the onset and progression of type 2 diabetes.
Chapter 2: Tubulin-derived Peptides as Imaging Agents to Image Highly Invasive Breast Cancer Subsets

2.1 Introduction

2.1.1 Hyaluronan, RHAMM and Breast Cancer

Breast cancer is the most common cancer in women over the age of twenty and is a major cause of mortality worldwide. Figure 2.1 shows the number of prevalent cases for the leading types of cancer in Canada, with breast and prostate being the most prevalent for all three lengths of cancer duration.62

![Figure 2.1 Number of prevalent cases of leading cancers, Canada, 2005](image)

Every day, 62 Canadian women are diagnosed and 14 Canadian women die of the disease.63 Breast cancer is a heterogeneous disease and as a result, it becomes challenging to manage. It contains subpopulations of aggressively invasive, highly proliferative tumour cells as well as slowly proliferating tumour initiating cells that are resistant to chemotherapy.64 Consequently, treatment regimens need to be personalized to individual patients in order to be successful. The current screening techniques are not
adequate. Mammography is one of the present screening methods for breast cancer, although the accuracy remains quite low.\textsuperscript{65} Often the primary tumour is too small to be clinically detected, and once it is large enough, it may have progressed into metastasis in some patients. Metastasis is the final step in tumour progression and is defined as the process of tumour cells leaving their niche, colonizing distant organ sites and forming secondary lesions.\textsuperscript{66} There is a great need for earlier detection methods in order to aid in the detection and treatment of metastatic breast cancer. Molecular imaging techniques can be very useful for screening, staging, response evaluation and guided surgery, radiotherapy and systemic treatment.\textsuperscript{67}

Tumour cells attach and migrate along the extracellular matrix which acts as a physical scaffold to help regulate cell motility, proliferation, invasion and metastasis.\textsuperscript{68} Hyaluronan is a component of the extracellular matrix and its accumulation is a prognostic indicator of poor outcome in breast as well as other cancers. Hyaluronan (HA) is a negatively charged high molecular weight linear polysaccharide consisting of repeating units of D-glucuronic acid and N-acetyl glucosamine (Figure 2.2).\textsuperscript{69}

\textbf{Figure 2.2} Structure of hyaluronan\textsuperscript{69}

HA is essential for proper cell growth, organ structural stability and tissue organization.\textsuperscript{70} It performs these cellular processes through its interaction with receptors on the cell surface, a family of proteins termed hyaladherins. There are three main groups of cell receptors for HA: cluster designation 44 (CD44), intracellular adhesion molecule-1 (ICAM1) and receptor for hyaluronan mediated motility (RHAMM). CD44 is
the best characterized HA receptor and is essential to the functioning of normal cells, but is also expressed in many malignant cell types.\textsuperscript{69, 71} It is part of the link module family of hyaladherins which all share a link fold defined by two α helices and two triple-stranded antiparallel β sheets.\textsuperscript{72} However, not all HA receptors contain a link module, such as RHAMM which bears no similarity in secondary structure to other HA binding proteins. Full length RHAMM is 85 kDa and its two predicted HA binding domains are contained within separate highly basic coiled regions that flank one another about a central groove.\textsuperscript{73} It binds to HA on the cell surface and regulates cell proliferation and promotes cell locomotion.\textsuperscript{74} The appealing quality of RHAMM is that it is poorly expressed in normal cells,\textsuperscript{75} transiently expressed following tissue injury and is highly over-expressed in metastatic cancers and implicated in tumour metastasis, specifically in cell motility and invasion.\textsuperscript{76} The HA binding motif of RHAMM has been identified near the carboxy-terminus and consists of basic residues\textsuperscript{8}. The region has a BX\textsubscript{2}B motif, where B is a basic residue and X is often a hydrophobic residue, but may be any residue excluding acidic.\textsuperscript{73} This motif is common to many proteins, so is not reliable for confirmation of HA binding. Nonetheless, it is evident that the main interaction between RHAMM and HA is likely based upon ionic interactions and the spatial distribution of the positively charged residues in RHAMM and the negatively charged carboxylate groups in HA to stabilize the complex. In addition, there are likely small contributions from hydrophobic interactions involving lipophilic patches on RHAMM and π-interactions between aromatic residues. Non-polar side chains may be involved in positioning the negative charges of the ligand so they interact with the corresponding positive charges on RHAMM.\textsuperscript{73} Previously, HA was developed into an imaging agent and labelled with \textsuperscript{99m}Tc. Under homeostatic conditions, it was found to target the HA receptor for endocytosis (HARE) which is located in the liver.\textsuperscript{61} The HARE receptor is responsible for the clearance of HA
and other glycosaminoglycans.\textsuperscript{77} The largest issue with HA as an imaging agent is that it has the potential to bind to any of the HA receptors, and not specifically to RHAMM. Moreover, HA would be rapidly degraded by hyaluronidase enzymes (Hyal-1, Hyal-2, PH-20).\textsuperscript{78, 79} In order to develop a RHAMM specific imaging agent that will not be degraded by hyaluronidases, new ligands are desired.

\subsection*{2.1.2 Tubulin-Derived Peptides Targeting RHAMM}

In addition to the cell surface, RHAMM is also reported to localize in the cytoplasm in a series of human breast cancer and various rodent tumour cell lines.\textsuperscript{80} It localizes at the perinuclear microtubule network during interphase and appears to control proliferation and gene expression required for cell cycle progression.\textsuperscript{77} This localization to microtubules is mediated by the carboxy terminus of RHAMM. Microtubules are structures that maintain cell morphology and mediate functions such as mitosis and locomotion, which require interaction between microtubules and microtubule associated proteins (MAPs).\textsuperscript{81} Microtubules consist of \(\alpha\beta\)-tubulin dimers whose carboxy terminal region contains a hypervariable carboxy terminal tail (CTT) composed of acidic residues. It is especially rich in glutamic acid residues and is likely to project outward from the rest of the molecule because of the electrostatic repulsion among the high negative charge density.\textsuperscript{82} The three-dimensional structure of the globular domain of microtubules has been determined by x-ray crystallography, however, the heterogeneous C-terminal region has only been predicted by computer modeling.\textsuperscript{83} Both \(\alpha\)- and \(\beta\)-tubulin consist of various isotypes that differ in their amino acid sequence and cellular and tissue distribution.\textsuperscript{84} For the assembly of microtubules to occur, the CTT must first be removed from tubulin by the serine protease, subtilisin, which cleaves the
C-terminal 10-20 amino acids of α- and β-tubulin. MAPs are positively charged and bind to the negatively charged CTT region of α- and β- tubulin.

2.1.3 Rational Approach for the Design of Peptides

In the design of peptide mimics, there are two broad approaches. The first is the use of a random combinatorial peptide library and the second is a rational based design. In this case a rational approach was used since the structure of the target is known. This approach can be further specified into a ligand-based design. In this design, targeting entities are developed with regard to the structure of the target receptor and what ligands may potentially bind to it. In order to predict potential ligands that will target the choice receptor, often bioinformatics is used. As a result, information regarding the target protein structure/sequence is required for this approach. Bioinformatics is a very useful discipline that uses many areas of mathematics, computer science and engineering to process biological data. Sequence alignment tools such as BLAST (basic local alignment search tool) allow for protein sequences to be screened to large online databases and identify proteins that show a high level of sequence similarity. Programs, such as ClustalX2, can make pairwise comparisons and calculate homology between the sequences found using BLAST and the target sequence. The Clustal series provide robust, portable programs that afford biologically accurate alignments within a reasonable time.

Using BLAST and ClustalX2, database searches between RHAMM and microtubule binding domain of MAPs revealed only a moderate sequence homology of 17-24% to the HA binding domain of RHAMM. More importantly, both RHAMM and MAPs have a stretch of basic residues. Furthermore, the secondary structures of the tubulin binding sites of MAPs and hyaluronan binding site of RHAMM have the same
degree of helicity and both could be classified as a basic-zipper domain. Since MAPs show direct binding to CTTs of tubulin, the hypothesis is that RHAMM should interact with synthetic peptides representing the CTTs of tubulin subtypes. The objective of this thesis is for the discovery of low molecular weight ligands that strongly and specifically target RHAMM for diagnostic and therapeutic purposes. Our goal is to develop a peptide-based imaging agent that mimics hyaluronan (HA) to address two obstacles in clinical studies: early detection and tumour cell heterogeneity. A molecular imaging probe that strongly targets the HA receptor RHAMM will permit the selective detection of highly tumorigenic progenitor cells in primary and metastatic carcinoma. Furthermore, the non-invasive imaging of RHAMM post-chemotherapy has the potential to determine residual disease. A second goal is to improve the in vivo stability of the peptides and potentially the affinity to RHAMM by incorporating unnatural amino acids.

2.2 Previous Research

Various peptides were synthesized for this study based off of tubulin fragments taken from the CTT region or directly flanking it (Table 2.1). Peptides were chosen based on the most common tubulin isoforms, as well as the most acidic region of the peptide. The seventeen tubulin derived peptides were evaluated using surface plasmon resonance (SPR). For this experiment, RHAMM was immobilized to the SPR sensor chip surface, and the tubulin peptides were injected and passed across the RHAMM surface. The real time association and dissociation interactions of each peptide to RHAMM are detected and reported as sensograms. The resulting sensograms measure the affinity of each individual peptide to the HA binding region of RHAMM. The results highlight the discovery of six high affinity peptides (Table 2.2).
Table 2.1 Sequences from fragments of various tubulin isoforms\textsuperscript{93}

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Tubulin Fragment</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSADGEDGEYY</td>
<td>αlc (438-449)</td>
<td>CTT</td>
</tr>
<tr>
<td>VEVEGEDEEGEEY</td>
<td>αla (440-451)</td>
<td>CTT</td>
</tr>
<tr>
<td>SVEAEAEDEEGY</td>
<td>αllc (439-450)</td>
<td>CTT</td>
</tr>
<tr>
<td>IDSVEDEDEEGY</td>
<td>αlVa (437-448)</td>
<td>CTT</td>
</tr>
<tr>
<td>DSFENEVEEGEEF</td>
<td>αVIII (438-449)</td>
<td>CTT</td>
</tr>
<tr>
<td>LKEKYYEGVEGDS</td>
<td>αla (428-439)</td>
<td>H12</td>
</tr>
<tr>
<td>GEFSEARDMAA</td>
<td>αla (416-427)</td>
<td>H12</td>
</tr>
<tr>
<td>FVHWYVGMEEMEE</td>
<td>αla (404-415)</td>
<td>CTT</td>
</tr>
<tr>
<td>GEEVEEDEEA</td>
<td>βlla (434-445)</td>
<td>CTT</td>
</tr>
<tr>
<td>EEDFEEVEEAAA</td>
<td>βla (433-444)</td>
<td>CTT</td>
</tr>
<tr>
<td>GEEVEEEVEEA</td>
<td>βIV (433-444)</td>
<td>CTT</td>
</tr>
<tr>
<td>EAFEDEEEIDG</td>
<td>βVI (435-446)</td>
<td>CTT</td>
</tr>
<tr>
<td>SNMNDLVESEYQD</td>
<td>βllla (413-424)</td>
<td>CTT</td>
</tr>
<tr>
<td>FTEAESENMNDLV</td>
<td>βllla (408-419)</td>
<td>CTT</td>
</tr>
<tr>
<td>RPDPDSTMGTQEO</td>
<td>γl (440-451)</td>
<td>CTT</td>
</tr>
<tr>
<td>VQQLIDYEHAAT</td>
<td>γl (428-439)</td>
<td>CTT</td>
</tr>
<tr>
<td>DNPDEMDSREI</td>
<td>γl (416-427)</td>
<td>CTT</td>
</tr>
</tbody>
</table>

Table 2.2 Average $K_D$ values of peptides with affinity for RHAMM from SPR screen\textsuperscript{93}

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Average $K_D$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVEAEAEEGEEY-NH$_2$</td>
<td>331.1 ± 24.5</td>
</tr>
<tr>
<td>GEEVEEVEEVEA-NH$_2$</td>
<td>130.0 ± 12.9</td>
</tr>
<tr>
<td>VEVEGEDEEGEEY-NH$_2$</td>
<td>24.2 ± 0.4</td>
</tr>
<tr>
<td>EEDFEEVEEAAA-NH$_2$</td>
<td>32.6 ± 1.1</td>
</tr>
<tr>
<td>EAFEDEEEEDG-NH$_2$</td>
<td>211.3 ± 8.6</td>
</tr>
<tr>
<td>FTEAESNMNDLV-NH$_2$</td>
<td>30.2 ± 1.5</td>
</tr>
</tbody>
</table>
In order to determine the selectivity of the peptides to the HA binding domain of RHAMM, a competitive ELISA was performed. The peptides were labelled with FITC on the N-terminus as shown in Figure 2.3. Binding is thought to occur at the C-terminus so all modifications were done on the N-terminus.

Figure 2.3 Peptide modified with aminohexanoic acid linker and fluorescein isothiocyanate onto the N-terminus

Fluorescein-labelled peptides are added to ELISA plates containing immobilized RHAMM and increasing concentrations of HA are added. If there is a competition for the binding site, a concentration dependent decrease in fluorescence will occur as the concentration of HA increases and displaces the peptides. The results indicate that four of the candidates were able to compete with HA for binding.93

Figure 2.4 ELISA assay of fluorescein-labelled peptides immobilized to recombinant RHAMM and CD44. Displacement of peptides by HA were seen for RHAMM, but not CD44.93
To confirm the specificity of the tubulin peptides to RHAMM, their affinities were tested against both RHAMM and CD44, a second receptor for HA (Figure 2.4). There is evidence that many of the peptides are interacting with RHAMM, but not CD44. From the results, three candidate peptides were chosen based on their specificity and high affinity to the HA binding domain of RHAMM (Figure 2.5).

1. VEGEGEEGEY-NH$_2$  \( K_D = 24 \text{ nM} \)
2. EEDFGEEAEAA-NH$_2$  \( K_D = 32 \text{ nM} \)
3. FTEAESNMNLVD-NH$_2$  \( K_D = 30 \text{ nM} \)

Figure 2.5 Selective, high-affinity ligands for RHAMM

### 2.3 Project Goal

It is now proposed that these peptide ligands, when modified to incorporate a positron-emitting isotope, will be suitable for the non-invasive imaging of RHAMM expression in vivo using PET. Thus, the molecular imaging probes are being developed in order to aid in the early and selective detection of tumorigenic progenitor cells in primary and metastatic carcinoma by specifically targeting RHAMM.

### 2.4 Results and Discussion

#### 2.4.1 Synthesis of Imaging Agents

The design of peptide-based molecular imaging probes often involves the addition of a metal chelation system. A typical compound used to generate these chelation systems is 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). In order to modify the peptides with DOTA, each peptide was first synthesized containing a linker, aminohexanoic acid (Ahx), on the N-terminus. The purpose of the linker is to
reduce any steric effects imposed by the macrocyclic DOTA ring on the binding of the peptide chain. The peptides were synthesized using standard Fmoc solid phase peptide synthesis (SPPS) methods either manually or with an automated synthesizer (Scheme 2.1).

Scheme 2.1 Solid phase peptide synthesis

N-Fmoc removal was achieved using 20% v/v piperidine in DMF, and the Kaiser test is performed to confirm the presence of free primary amino groups. Amino acids with reactive side chains were protected with acid labile orthogonal protecting groups such as OtBu and tBu. A representative synthetic route is described in Scheme 2.2, illustrating the preparation of DOTA-Ahx-peptide-NH₂. Typically in our lab when DOTA is desired as a chelator, it is bought commercially in the form of DOTA-tris-t-butyl ester and is coupled to the peptide under normal coupling conditions. However, this is usually quite expensive and a much cheaper alternative is to synthesize DOTA directly onto the peptide. Since the binding to RHAMM is thought to occur at the C-terminus and modifications are made at the N- terminus of the peptide, the addition of the chelator during solid-phase synthesis was possible. Once the linker was coupled to the peptide (4), it was modified with a DOTA chelator in a three step process. The first step uses DIC to couple bromoacetic acid to the peptide (5). The next step is to react 5 with cyclen, displacing the Br leaving group, and producing 6. Finally, t-butyl bromoacetate is reacted with 6, then the peptide is cleaved from the resin and all protecting groups removed, including the t-butyl groups.
of DOTA, using trifluoroacetic acid (TFA) to produce the DOTA peptides, 7a, 8a and 9a. **Figure 2.6** shows the HPLC and MS traces for 7a and 9a while the values for the purity of all the peptides along with their respective ESI-MS characterization are given in **Table 2.3**.

![Scheme 2.2 Synthesis for the addition of DOTA chelator to a peptide](image-url)
Figure 2.6 A. HPLC trace and MS spectra for 7a. B. HPLC trace and MS spectra for 9a.ii

Table 2.3 Analysis of synthesized DOTA peptides using UHPLC and ESI-MS

<table>
<thead>
<tr>
<th>Compound</th>
<th>Peptide</th>
<th>Calculated m/z [M+2H]^{2+}</th>
<th>Observed m/z</th>
<th>Purity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>7a</td>
<td>DOTA-Ahx-VEGEGEEEGEEY-NH$_2$</td>
<td>927.4003</td>
<td>927.4018</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>8a</td>
<td>DOTA-Ahx-EEDFGEEAEEA-NH$_2$</td>
<td>941.3978</td>
<td>941.3815</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>9a.i</td>
<td>DOTA-Ahx-FTEAESNMNDLV-NH$_2$</td>
<td>934.4413</td>
<td>934.4376</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>9a.ii</td>
<td>DOTA-EEA-FTEAESNMNDLV-NH$_2$</td>
<td>950.4362</td>
<td>950.4348</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>7b</td>
<td>$^{69/71}$Ga-DOTA-Ahx-VEGEGEEEGEEY-NH$_2$</td>
<td>961.3524</td>
<td>961.3503</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>8b</td>
<td>$^{69/71}$Ga-DOTA-Ahx-EEDFGEEAEEA-NH$_2$</td>
<td>975.3499</td>
<td>975.3498</td>
<td>&gt;90%</td>
</tr>
<tr>
<td>9b</td>
<td>$^{69/71}$Ga-DOTA-EEA-FTEAESNMNDLV-NH$_2$</td>
<td>984.3882</td>
<td>984.3896</td>
<td>&gt;90%</td>
</tr>
</tbody>
</table>

*Purity is determined through the integration of the area under the curve

Before a radionuclide is incorporated, it must first be characterized with a cold,
non-radioactive isotope. Two of the candidate peptides (7a and 8a) were labeled with cold $^{68/71}$Ga (Scheme 2.3).

![Scheme 2.3](image)

**Scheme 2.3** Synthesis for the incorporation of cold $^{69/71}$Ga to a DOTA peptide

A buffer is needed to keep the desired pH of 4 since under basic conditions, it will form insoluble gallate ions [Ga(OH)$_4$]$^-$. $^9$a.i was insoluble in the buffer, and as a result was unable to be cold labeled. Instead, a hydrophilic linker was added in place of aminohexanoic acid in hopes of increasing the solubility in aqueous buffer. The peptide containing the mini PEG linker, AEEA (2-[2-(2-aminoethoxy)ethoxy]acetic acid), $^9$a.ii was still not soluble and needed a 1:1 ratio ethanol: sodium acetate buffer in order to fully dissolve. After the reaction was complete for 7a, 8a and 9a.ii, the mixture was cooled and was purified by a C18 RP Sep-Pak. The peptides, 7b, 8b and 9b were characterized using LC/MS (Table 2.3).

### 2.4.2 $^{68}$Ga labelling

Since the cold $^{69/71}$Ga labeling was successful, the DOTA peptides, 7a and 8a were then radiolabelled with $^{68}$Ga using HEPES buffer and heated at 80°C for 30 minutes. The radiolabelling of compounds was carried out using an Eckert and Ziegler $^{68}$Ge/$^{68}$Ga generator connected to a series of synthesis modules, which are remotely controlled by the user via a process computer (Figure 2.7). A graphical representation of the program
used for radiolabelling peptides using $^{68}$Ga is shown in Figure 2.8 as well as the various components in Table 2.4.

**Figure 2.7** A. Eckert and Ziegler modular lab automated synthesis unit for $^{68}$Ga labelling; B. Polymeric strata used as part of the automated synthesis unit for $^{68}$Ga labelling

**Figure 2.8** Schematic of a program designed for the $^{68}$Ga-radiolabelling of peptides
Table 2.4 Description of components used in Figure 2.8

<table>
<thead>
<tr>
<th>Component</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vial</td>
<td><img src="image" alt="Vial" /></td>
</tr>
<tr>
<td>Squeeze pump</td>
<td><img src="image" alt="Squeeze pump" /></td>
</tr>
<tr>
<td>Cation exchange column/sep pak</td>
<td><img src="image" alt="Cation exchange column/sep pak" /></td>
</tr>
<tr>
<td>Heating reaction vial</td>
<td><img src="image" alt="Heating reaction vial" /></td>
</tr>
<tr>
<td>Three-way valves</td>
<td><img src="image" alt="Three-way valves" /></td>
</tr>
<tr>
<td>Sterile filters</td>
<td><img src="image" alt="Sterile filters" /></td>
</tr>
</tbody>
</table>

All solutions in this system, with the exception 0.1 N HCl which is transferred by a pressure pump, are transferred via pressure variance. First, gallium-68 is eluted from the generator using a squeeze pump and 3 mL of 0.1 N HCl (vial 1-1). This eluate, which may contain impurities such as $^{68}$Ge breakthrough is then loaded onto a strong cation exchange column for initial purification. Gallium-68 is then eluted out of the cation exchange cartridge using 0.5 mL of 0.05 N HCl in 98% acetone (vial 1-3), and carried to the reaction vessel containing the peptide dissolved in buffer. After the reaction is complete, the mixture is passed through a C18 light Sep Pak for purification. A 2 mL aliquot of water is then passed through this cartridge to elute free Ga-68. The final product is isolated and eluted using EtOH into a clean glass vessel. A small sample of the final product is diluted with water prior to analysis by HPLC.
Peptides 7a and 8a were successfully radiolabelled with the positron emitting isotope $^{68}$Ga using 100 µg of precursor, in a radiochemical yield of 67% and 65%, respectively and radiopurity of $>$99% (Table 2.5: 7c., 8c). It is impossible to purify using HPLC because the unlabelled precursor elutes very close compared to the gallium coordinated peptide, as seen in Figure 2.9, and is the same case for all the peptides.

![Stacked HPLC traces of the precursor, 7a, cold $^{69/71}$Ga coordinated peptide, 7b, and $^{68}$Ga labelled peptide, 7c](image)

Figure 2.9 Stacked HPLC traces of the precursor, 7a, cold $^{69/71}$Ga coordinated peptide, 7b, and $^{68}$Ga labelled peptide, 7c

In order to improve specific activity and still obtain approximately the same radiochemical yield, experiments were performed to decrease the amount of precursor needed. It was found that the amount of precursor can be decreased to 10 µg while still obtaining reasonable yields. In this method, the automated system was not used, and instead the peptide was reacted with $^{68}$Ga manually. The reaction took place at 90°C for 10 minutes. After the reaction, it was passed through an equilibrated C18 RP light Sep-Pak, flushing with water into the waste and ethanol to elute the product, respectively. 7a was reacted manually with $^{68}$Ga successfully to produce 7c in a radiochemical yield of 60 %, an estimated specific activity of $>$14 GBq/µmol and radiopurity of $>$99% (Table
2.5: 7c). Optimization of the labelling procedure led to using 20 µg of precursor, resulting in an improved specific activity of >19 GBq/μmol and radiochemical yield of 75%. Similarly, 9a.ii was labelled with 68Ga manually and a very high radiochemical yield of 91% was obtained using 20 µg of precursor and a good estimated specific activity of >30 GBq/μmol.

Table 2.5 Optimization of labelling conditions for the radiolabelled peptides

<table>
<thead>
<tr>
<th>Compound number</th>
<th>68Ga-DOTA-Ahx-VEEGEEGEEY-NH2</th>
<th>68Ga-DOTA-Ahx-EEDFGEAEVEA-NH2</th>
<th>68Ga-DOTA-AEEA-FTEAESNMNDLV-NH2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor Amount (µg)</td>
<td>100</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Specific Activity (GBq/µmol)</td>
<td>&gt;2</td>
<td>&gt;19</td>
<td>&gt;19</td>
</tr>
<tr>
<td>RCY (%) decay corrected (d.c.)</td>
<td>67</td>
<td>60</td>
<td>75</td>
</tr>
<tr>
<td>Radiopurity (%)</td>
<td>&gt;99</td>
<td>&gt;99</td>
<td>&gt;99</td>
</tr>
</tbody>
</table>

2.4.3 In Vivo Studies

The first in vivo experiment was performed with the radioactive compound 7c (using 10 µg of precursor), which was injected through the tail vein of xenograft mice with MDA-MB-231 tumours in the fourth mammary gland. MDA-MB-231 cells are a human breast cancer cell line that has been shown to overexpress RHAMM. Afterwards, biodistribution studies were performed by Dr. Lihai Yu, but were inconclusive and further evaluation was required. For the next attempt of in vivo studies, the tumour model was changed to LR21 cells. It is a fibroblast line, specifically 10T1/2 cells, transfected with the v4 isoform of RHAMM. A pilot study was performed with three xenograft mice with tumours injected
into the flank. Since the radioactive compound 9c (20 µg of precursor) was resulting in a better specific activity than 7c, it was used for the study and was injected through the tail vein of the mice. The sacrifice of the mice followed by biodistribution was performed by Dr. Lihai Yu two hours after injection. The results demonstrate that the probe did not target the tumour. All organs showed no significant uptake excluding the kidneys and tail, where the probe was excreted and injected, respectively. It is evident that the probes are not adequate imaging agents in vivo in the models tested. It is possible the peptides may not be stable in vivo and are being degraded.

2.4.4 Design of Peptide Analogues

In order to optimize the affinity of the peptides for RHAMM, we performed an alanine scan to determine which residues are critical for peptide-receptor interaction. The technique uses the amino acid, alanine, to substitute each residue in the sequence sequentially and the fluorescence is measured. Peptides were synthesized with a linker and a fluorescein label attached to the N-terminus. The alanine scans for peptides 21 and 22 were previously performed in which the important amino acids are indicated (Figure 2.10). The remaining peptide, 2, needed to be synthesized with FITC and the linker as well as its analogues with the sequential substitution of alanine. However, the scan showed no key amino acids and was re-tested for confirmation. Table 2.6 shows the sequence of the eleven synthesized peptides as well as their calculated and observed masses.
Figure 2.10 Alanine scans for peptides FTEAESNMNDLV (left) and VEGEGEEEGEEY (right). The stars represent the sequences where Ala has replaced a key amino acid.

Table 2.6 Mass spectrometric analysis of synthesized peptides

<table>
<thead>
<tr>
<th>Compound</th>
<th>Peptide</th>
<th>Calculated</th>
<th>Observed</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[M+2H]^2+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Fitc-Ahx-EEDFGEEAEAAA-NH2</td>
<td>942.8</td>
<td>943.6</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>11</td>
<td>Fitc-Ahx-ΑEDFGEEAEAAA-NH2</td>
<td>913.8</td>
<td>914.4</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>12</td>
<td>Fitc-Ahx-EADFGEAEAAA-NH2</td>
<td>913.8</td>
<td>914.5</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>13</td>
<td>Fitc-Ahx-EEAFGEAEAAA-NH2</td>
<td>920.8</td>
<td>921.4</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>14</td>
<td>Fitc-Ahx-EEDAGEEAEAAA-NH2</td>
<td>904.8</td>
<td>905.4</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>15</td>
<td>Fitc-Ahx-EEDFAEEAEAAA-NH2</td>
<td>949.9</td>
<td>950.6</td>
<td>&gt;90%</td>
</tr>
<tr>
<td>16</td>
<td>Fitc-Ahx-EEDFGAEAEAAA-NH2</td>
<td>913.8</td>
<td>914.5</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>17</td>
<td>Fitc-Ahx-EEDFGEAEAEAAA-NH2</td>
<td>913.8</td>
<td>914.5</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>18</td>
<td>Fitc-Ahx-EEDFGEEAEAAA-NH2</td>
<td>913.8</td>
<td>914.4</td>
<td>&gt;90%</td>
</tr>
<tr>
<td>19</td>
<td>Fitc-Ahx-EEDFGEAEAEAAA-NH2</td>
<td>913.8</td>
<td>914.4</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>20</td>
<td>Fitc-Ahx-EEDFGEAEAAA-NH2</td>
<td>913.8</td>
<td>914.4</td>
<td>90%</td>
</tr>
</tbody>
</table>

After the alanine scans were prepared for peptides 21 and 22, truncation studies must be designed and performed to ensure the peptides contain the key amino acids that were shown in Figure 2.12. For initial studies, two truncated versions of peptide 20 and one version of peptide 21 were synthesized. In addition to the truncated peptides,
positive and negative controls were also synthesized, and will be evaluated using ELISA with RHAMM acting as the blank (Table 2.7).

**Table 2.7** Alanine scan results and subsequent truncated sequences and controls that will be evaluated using ELISA

<table>
<thead>
<tr>
<th>#</th>
<th>Peptide Sequence</th>
<th>Important Amino Acids</th>
<th>Truncated Sequence</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>FITC-Ahx-FTEAESNMNDLV-NH₂</td>
<td><em>TE</em>ES<strong>ND</strong></td>
<td>TEAES-NH₂</td>
<td>-ve: SAETE-NH₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ESNAND-NH₂</td>
<td>(scrambled sequence)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-ve: NDASEN-NH₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(scrambled sequence)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+ve: FTEAESNMNDLV-NH₂</td>
</tr>
<tr>
<td>22</td>
<td>FITC-Ahx-VEGEGEEEGEEY-NH₂</td>
<td><em>E</em>E**EEGE*Y</td>
<td>EEEGAY-NH₂</td>
<td>-ve: EYAEGE-NH₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(scrambled sequence)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+ve: VEGEGEEGEEY-NH₂</td>
</tr>
</tbody>
</table>

Unfortunately, purified recombinant RHAMM protein is needed for the ELISA and was not synthesized in time so there are currently no results to report.

Since it is unknown how truncation affects the affinity of the peptides, alternatively amino acid modifications were performed. This was done in order to further optimize the affinity and help improve the *in vivo* stability of the peptides through the use of unnatural amino acids. The important amino acids, determined from the alanine scans, were individually replaced by unnatural amino acids and bioisosteres. According to Burger, bioisosteres are defined as compounds that possess near-equal molecular shapes and volumes, approximately the same distribution of electrons and exhibit similar physical properties. The objective of the replacement is to enhance either the desired physical or biological properties without altering the chemical structure significantly. There are classical and nonclassical bioisosteres. Classical bioisosteres involve structurally simple groups such as monovalent atoms (D, F), divalent atoms (C=O, C=S)
or ring equivalents. In contrast, nonclassical bioisosteres are structurally distinct, and often exhibit different steric and electronic properties.\textsuperscript{96}

In the literature, there are several examples of bioisosteres of carboxylic acids and amides,\textsuperscript{96-99} however, due to time constraints it was opted to only pursue commercially available compounds. As a result, many of the more common and interesting bioisosteres, such as tetrazoles, sulfonamides, oxazoles and a trifluoroethylamine moiety were not used. Instead, an acidic sulfonate was used to mimic the carboxylic acid of glutamic and aspartic acid in the form of cysteic acid. To mimic asparagine, a couple different replacements were attempted. The first replacement utilized an ester instead of the amide (Asp-OMe-OH). In the second replacement, the asparagine was replaced with a thiazole (Ala-4-thiazoyl-OH). Glycine, being structurally very simple could not be changed very much except for the addition of methyl (methylene) groups at varying positions. Some of the more obvious replacements were used for serine and threonine such as replacing the hydroxy group with a methoxy or adding a methyl group onto the peptide backbone. Similarly, the main replacements for tyrosine were replacing the hydroxy group with various other groups such as an amine or methoxy as well as changing the aromatic group to a bulkier naphthalene.

The amino acids considered not to be critical were individually replaced with amino acids containing various groups such as an aromatic or an alcohol to determine if the affinity can be improved. Furthermore, each amino acid, critical or noncritical, was replaced with its D-amino acid counterpart to incorporate unnatural amino acids into the sequence and vary its conformation. Table 2.8 lists all of the critical amino acid replacements and Table 2.9 lists all other amino acid replacements for the peptides 1 (VEGEgEEGEgEEY) and 3 (FTEAESNMNDLV).
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Bioisosteric Replacement</th>
<th>Amino acid replacement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser, Thr</td>
<td>OH to OMe (only Ser) β-HSer, β-HThr Me to peptide backbone</td>
<td>D-Ser D-Thr</td>
</tr>
<tr>
<td>Asn</td>
<td>Amide to: ester, thiazole,</td>
<td>Gln D-Asn</td>
</tr>
<tr>
<td>Glu, Asp</td>
<td>COOH to sulfonate</td>
<td>D-Glu, D-Asp</td>
</tr>
<tr>
<td>Tyr</td>
<td>OH to: F, OMe, NH₂ NO₂ Nal</td>
<td>Phe D-Tyr</td>
</tr>
<tr>
<td>²⁵Gly</td>
<td>β-Ala Aib</td>
<td>D-Ala</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Amino Acid Replacement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe</td>
<td>Tyr, Nal, Ala, Ser, Asp D-Phe</td>
</tr>
<tr>
<td>Ala</td>
<td>Aib, Ser, Phe D-Ala</td>
</tr>
<tr>
<td>Asn</td>
<td>Gln, Asp, Leu, Tyr D-Asn</td>
</tr>
<tr>
<td>Met</td>
<td>Val, Phe, Nle, Thi D-Met</td>
</tr>
<tr>
<td>Leu</td>
<td>Val, Ser, Phe D-Leu</td>
</tr>
<tr>
<td>Val</td>
<td>Ala, Ile, Phe, Asp D-Val</td>
</tr>
<tr>
<td>²⁵Gly</td>
<td>Ala, Aib, β-Ala D-Ala</td>
</tr>
<tr>
<td>Glu</td>
<td>Ala, Asp, Gln, Thr, Phe D-Glu</td>
</tr>
</tbody>
</table>

The peptides were synthesized on tentagel S NH₂ resin on the tip synthesis workstation (Biotage, Uppsala, Sweden). The tip station allows for the synthesis of many peptides simultaneously in small amounts. The tip module offers wells for 96 peptides and the amino acid rack offers space for 25 different amino acids. Unfortunately, the synthesis had to be broken into three separate syntheses due to the limited rack space (25 slots).
to hold the number of unnatural amino acids (28) and natural amino acids (12) adding to a total of 40 amino acids. For the screening of the peptides against RHAMM, it is necessary that they are stable against the acidic conditions. Tentagel resin was used instead of Rink amide resin because of the PEG linker it possesses and the ability for biological evaluation in aqueous media. Tentagel contains a free amine and forms a stable amide bond with the peptide. Therefore the peptides are deprotected during the acidic cleavage conditions, but remain on resin. All the peptides synthesized for this experiment are shown in Table 2.10, including the positive control peptides with no changes made and a scrambled version of each as the negative controls. Before the analogues were synthesized, several test peptides were synthesized on Rink amide resin in order to confirm that an acceptable purity is obtained since this will be the first time the tip synthesizer is used. One of the amino acids, Fmoc-cysteic acid-OH had to first be modified before further use since in its commercial form, it is not soluble in DMF or NMP (N-methyl-2-pyrrolidone). Since it is unknown whether the commercial form was the free acid or possessed a Na\(^+\) counterion, we erred on the cautious side and dissolved the amino acid in water and ran the solution through an ion exchange column (Amberlite IR-120H). It was then lyophilized and the free acid was treated with 1.05 eq of 40% tetrabutylammonium hydroxide (aq) and was lyophilized. The resultant white, glassy solid was highly soluble in DMF and was used as part of the synthesis of the peptides involving cysteic acid on the tip workstation.

\[
\begin{align*}
\text{Fmoc-L-Cys(\text{O}_3\text{Na})-OH} & \xrightarrow{\text{Amberlite IR-120H}} \text{Fmoc-L-Cys(\text{O}_3\text{H})-OH} & 1.05 \text{ eq Bu}_4\text{NOH} & \xrightarrow{} \text{Fmoc-L-Cys(\text{O}_3\text{NBu}_4)-OH}
\end{align*}
\]

**Scheme 2.4** The synthesis of the tetrabutylammonium salt of cysteic acid
Table 2.10 Peptides synthesized on tip synthesizer for screen. Resin was tentagel S NH₂ (90 μm). Scale = 0.0005 mmol

<table>
<thead>
<tr>
<th>D-amino acids</th>
<th>FTEAESNMNDLV (+ve control)</th>
<th>VEGEGEEE (ve control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SVNLEFMANEDT (- ve control)</td>
<td>GYGEESV (ve control)</td>
</tr>
<tr>
<td>FTEA(Ser-OMe)NMNDLV</td>
<td>VEGEGEEE (Phe-4-F)</td>
<td></td>
</tr>
<tr>
<td>FTEA(β-HSer)NMNDLV</td>
<td>VEGEGEEE (TyrMe)</td>
<td></td>
</tr>
<tr>
<td>F(β-HThr)EAESNMNDLV</td>
<td>VEGEGEEE (Phe-4-NH-Boc)</td>
<td></td>
</tr>
<tr>
<td>FTEAO(β-Me-Ser)NMNDLV</td>
<td>VEGEGEEE (Phe-4-N02)</td>
<td></td>
</tr>
<tr>
<td>F(β-Me-Thr)EAESNMNDLV</td>
<td>VEGEGEEEnal</td>
<td></td>
</tr>
<tr>
<td>FTEAESNMQDLV</td>
<td>VEGEGEEAibEEY</td>
<td></td>
</tr>
<tr>
<td>FTEAESNM(D-OMe)DLV</td>
<td>VEGEGEE(β-Ala)EEY</td>
<td></td>
</tr>
<tr>
<td>FT(CA)AESNMNDLV</td>
<td>V(CA)EGEGEEE</td>
<td></td>
</tr>
<tr>
<td>FTEA(CA)SNMNDLV</td>
<td>VEG(CA)EGEGEEE</td>
<td></td>
</tr>
<tr>
<td>FTEAESNM(CA)LV</td>
<td>VEGE(CA)EGEGEEE</td>
<td></td>
</tr>
<tr>
<td>CA=cysteic acid</td>
<td>VEGE(CA)EGEGEEE</td>
<td></td>
</tr>
<tr>
<td>FTERAESNMNDLV</td>
<td>VEAlbEGEGEEE</td>
<td>VEAlbEGEGEEE</td>
</tr>
<tr>
<td>VTTERAESNMDLV</td>
<td>VEAlbE(β-Ala)EEY</td>
<td>VEAlbE(β-Ala)EEY</td>
</tr>
<tr>
<td>FTEAibESNMNDLV</td>
<td>IEGEGEEE</td>
<td></td>
</tr>
<tr>
<td>FTEAESNMNDLV</td>
<td>TEGEGEEE</td>
<td></td>
</tr>
<tr>
<td>FTEAESNMNDLV</td>
<td>FEGEGEEE</td>
<td></td>
</tr>
<tr>
<td>FTEAESOMNDLV</td>
<td>VEGEGEEE</td>
<td></td>
</tr>
<tr>
<td>FTEAESDMNDLV</td>
<td>VEGEGEEE</td>
<td></td>
</tr>
<tr>
<td>FTEAESLMNDLV</td>
<td>VEGEGTEE</td>
<td></td>
</tr>
<tr>
<td>FTEAESYMNLDLV</td>
<td>VEGEGEY</td>
<td></td>
</tr>
<tr>
<td>FTEAESYNVNDLV</td>
<td>VEGEGEEAY</td>
<td></td>
</tr>
<tr>
<td>FTEAESNNDLV</td>
<td>VEGEGEEEY</td>
<td></td>
</tr>
<tr>
<td>FTEAESNDLVL</td>
<td>VEGEGEEESY</td>
<td></td>
</tr>
<tr>
<td>FTEAESNENDLV</td>
<td>VEGEGEEEDY</td>
<td></td>
</tr>
<tr>
<td>FTEAESNNeNDLV</td>
<td>VEGEGEEEF</td>
<td></td>
</tr>
<tr>
<td>FTEAESNThiNDLV</td>
<td>FTEAESNMDSV</td>
<td></td>
</tr>
<tr>
<td>FTEAESNMDVF</td>
<td>FTEAESNMDLV</td>
<td></td>
</tr>
<tr>
<td>FTEAESNMDV</td>
<td>FTEAESNMLA</td>
<td></td>
</tr>
<tr>
<td>FTEAESNMDLT</td>
<td>FTEAESNMLY</td>
<td></td>
</tr>
<tr>
<td>FTEAESNMDLE</td>
<td>FTEAESNMDN</td>
<td></td>
</tr>
</tbody>
</table>
2.4.5 Library Screening

One method in discovering novel peptides is the one-bead one-compound (OBOC) approach. In an OBOC combinatorial library, the peptides consist of random sequences and are tethered to tentagel resin beads. Typically, the beads are incubated with fluorescent cells that express the protein of interest. Afterwards, the beads are screened using a plate reader or a biosorter to identify hit peptides with cells attached. This approach has been used to discover many novel peptides.\textsuperscript{101} In my case, the peptide library will consist of known rather than random sequences as in OBOC libraries. However, the same cell-based screening approach will be attempted to discover novel high affinity peptides. Before my peptide analogues were synthesized, the conditions for the on-bead cell binding assay needed to be determined. The assay was attempted with two of the lead peptides, 1 and 3 as well as scrambled versions of the peptides and beads with no peptide attached for negative controls in various cell lines that overexpress RHAMM. The three cell lines used in this experiment were the human breast cancer cell line MDA-MB-231 as well as a fibroblast line, 10T1/2 cells, that were transfected with RHAMM-V4, which can be further divided into two cell lines: LR21 and 10V4. The LR21 cells are a clonal cell line and have a homogenous level of RHAMM overexpression. The 10V4 cells are a mixture of clones, so they will have the cDNA integrated into different parts of the genome. Therefore the two exhibit the same characteristics, but LR21 are typically more aggressive compared with 10V4. A knockout (KO) cell line was also used which does not express any RHAMM, to act as a negative cell line. In all cases, the cells were dissociated, counted and approximately 200,000 cells were added to non-tissue culture plates. Approximately 3000 beads were added to each well with the cells, and the plates were incubated at 37°C for varying lengths of time (1-3 hours). Afterwards, the wells were washed with media (DMEM) several times.
and viewed using fluorescent microscopy. The LR21 and 10V4 cells seemed to work the best compared to the MDA-MB-231 cell line. As seen in Figure 2.11, the cells attached to the positive control beads (peptides 1 and 3) and not to the negative controls beads containing scrambled versions of peptides 1 and 3 or the knockout cells. Unfortunately, the cells did not bind to every positive bead and the ones that showed binding were not completely coated with cells as typically seen with integrin expressing cells. Also, it was at times difficult to judge if there was a real interaction occurring because the cells tend to clump together and in some cases may have been floating on top of the bead instead of bound to it.

![Images](image1.png)

**Figure 2.11** Images taken of the interactions between cell lines (green) interacting with positive and negative control beads (top images: blue; bottom images: grey). 

A. Magnified image of 10V4 cells binding to positive bead.  
B. Image of positive beads containing peptide 3 interaction with 10V4 cells. 
C. Image of beads containing a scrambled sequence (negative control) showing very little to no interaction with the 10V4 cells. 
D. Image of positive beads showing little to no interaction with the knockout cells. 
E. Image of positive beads containing peptide 1 interaction with LR21 cells

Since the results were not consistent for every positive bead and were not able to accurately be quantified, the on-bead cell screening assay was abandoned. The method itself has its own limitations as well, especially for an OBOC library. In an OBOC library,
the collection of hits by manual isolation of individual beads among millions under a microscope is labour intensive and time consuming. Overall, it is difficult to accurately assess the interaction of cells on a bead and manually separate the hits.

Alternatively, a “beads on a bead” screening approach has been developed which separates beads based on both magnetic and fluorescence sorting. This method is advantageous because it allows for magnetic bulk separation and afterwards fluorescence sorting. Furthermore, it allows for multiple proteins displayed on a fluorosphere. First, the target protein is biotinylated and is adsorbed to 2 µm streptavidin coated magnetic/fluorescent screening beads (Figure 2.12).

![Figure 2.12](image)

**Figure 2.12** The biotinylated protein is adsorbed to streptavidin coated magnetic and fluorescent screening beads

Upon mixing of the screening beads with the peptide library beads, they should associate with one another in an affinity-dependent manner. To validate the method, proof-of-principle experiments were previously performed by Cho *et al.* exploiting the binding of RGD containing peptides to αvβ3 integrin. Afterwards, the screening process was used to identify several high affinity 8-mer peptides for αvβ3 integrin from an OBOC library. The accurate and affordable strategy led us to adopt the method to screen the synthesized peptide analogues from the tip workstation to identify high affinity peptides with improved *in vivo* stabilities. The disadvantage in this method is that the native conformation of protein expressed at the cell surface can be very different compared to purified protein.
Due to the difficulty in producing pure RHAMM protein by recombinant methods, it was decided that a smaller section of RHAMM (7 kDa) containing the HA-binding domain, could be synthesized using solid-phase peptide synthesis. The 62 amino acid carboxy terminus of RHAMM containing the HA binding region was synthesized on the automated microwave synthesizer. This allows for the direct biotinylation of the N-terminal amine as part of the synthesis on-resin. The method avoids the use of kits and lengthy purification procedures to remove excess biotin. In addition, as biotin is only coupled to the N-terminus, it does not interfere with the multiple lysines in the sequence to interrupt any binding within the HA binding region. A linker was added in order to increase the solubility of biotin. The synthesized peptide containing biotin was HPLC purified and characterized (Table 2.11).

Table 2.11 Characterization of purified RHAMM

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Calculated</th>
<th>Observed</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin-AEEA-RDSYAQLLGHQNLKQIKHVVKLKDENSQKL</td>
<td>846.1069</td>
<td>846.0906</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>SEVSKLRLSQLVKRQNELRLQGELDALKGIR-NH₂</td>
<td>(average)</td>
<td>&gt;95%</td>
<td></td>
</tr>
</tbody>
</table>

Longer peptide sequences are difficult to synthesize and purify because of the accumulation of side reactions and unwanted by-products. More recently, microwave irradiation has emerged as a method to improve the efficiency of solid phase peptide synthesis. The heating capability allows for the reduction of inter- and intra-molecular derived aggregation and secondary structure formation (β-sheets) which results in greater product purity/yield as well as faster reaction times.\(^\text{103}\) 7 kDa RHAMM was synthesized on the microwave synthesizer in four separate syntheses, performing micro-cleaves after each synthesis in order to check the purity and determine problem
sections. Afterwards, the hydrophilic linker and biotin were added manually and the peptide was purified on the HPLC. The yield was sacrificed to obtain a high purity (>95%) product. To confirm that this shorter length of RHAMM is still capable of binding to HA, an ELISA was performed using an ECHELON kit containing HA-coated plates (Figure 2.13). Varying concentrations of biotinylated RHAMM were added to the plates and the binding was detected with streptavidin-AP. The results demonstrate that this shorter length RHAMM is capable of binding HA and is suitable for determining the affinity and potential of the 95 peptide analogues.

![Absorbance vs Concentration](image)

**Figure 2.13** ELISA of HA-coated plates with varying concentrations of biotinylated RHAMM

### 2.4.6 Addition of RHAMM to Magnetic/Fluorescent Beads

Before RHAMM was adsorbed to the magnetic and fluorescent streptavidin beads, the beads were washed with PBS to remove sodium azide. Washings were done holding a strong neodymium magnet to the side of the vial for several minutes while
pipetting the liquid out. The beads were then incubated with biotinylated RHAMM for 1.5 hours at room temperature. Biotin was then added into the solution and was incubated for an additional 30 minutes at room temperature to block any remaining streptavidin sites. To develop a procedure for screening the peptide analogues, first the control peptides were tested. The four controls (~0.5 mg) were incubated with the prepared RHAMM-coated magnetic/fluorescent beads (5 μL) (1.5 hr, 37°C). After the incubation period, the tentagel beads were washed five times to remove the unbound magnetic beads. To determine the limits for negative and positive hits, they were analyzed in a plate reader (ex 560 nm, em 590 nm) before incubation and after washing. This procedure was performed in triplicate to ensure its reliability. The fluorescent values were calculated as a ratio of total magnetic beads:bound beads left after washing and the values were normalized. The averages were calculated from normalized values and were set as the limits for positive and negative hits (Table 2.12).

Table 2.12 Synthesized controls for screening of analogues and their average normalized values from the fluorescent plate reader (560 nm, 590 nm)

<table>
<thead>
<tr>
<th>#</th>
<th>Peptide</th>
<th>Average Normalized Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>FTEAESNMNDLV (positive)</td>
<td>0.78</td>
</tr>
<tr>
<td>Scrambled 3</td>
<td>SVNLEFMANEDT (scrambled-negative)</td>
<td>0.23</td>
</tr>
<tr>
<td>1</td>
<td>VEGEGEEEVEGEY (positive)</td>
<td>0.51</td>
</tr>
<tr>
<td>Scrambled 1</td>
<td>GYGEEEEEVEGE (scrambled-negative)</td>
<td>0.38</td>
</tr>
</tbody>
</table>

The same procedure was carried out for the 95 synthesized analogues. Similarly, the ratio of total magnetic beads:bound beads was calculated for each peptide and the values were normalized and compared to the averages found for the controls. The screen resulted in several positive hits for both peptides (Table 2.13), however more were found for peptide 1 due to the lower limit for a positive hit.
Table 2.13 Sequences for positive hits with improved values over their respective positive control

<table>
<thead>
<tr>
<th>Original Sequence</th>
<th>FTEAESNMNDLV</th>
<th>VEGEGE\text{EE}GE\text{EE}Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequences of positive hits</td>
<td>FTEaESNMNDLV</td>
<td>VEGEGE\text{E}GE\text{E}Y</td>
</tr>
<tr>
<td></td>
<td>F(\text{\textbeta-}H\text{Thr})EAESNMNDLV</td>
<td>VEGEA\text{E}EE\text{E}GY</td>
</tr>
<tr>
<td></td>
<td>FTEAESNMN(\text{CA})LV</td>
<td>VEGEG\text{D}EE\text{E}GY</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VEGEGE\text{E}GE\text{AY}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VEGEGE\text{E}GE\text{E}N\text{al}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VEG(\text{CA})GEE\text{E}E\text{E}Y</td>
</tr>
</tbody>
</table>

Figure 2.14 Images of positive hits resulting from “beads on a bead” screen. Small red magnetic beads coating a tentagel bead containing a positive sequence

Some of the positive hits were viewed under a fluorescent microscope and images were taken (Figure 2.14). The results of the two replacements of glycine with alanine in peptide 1 are confirmed by the alanine scans. For example, there was a significant increase in the fluorescence for the replacement of G\text{11} with A using the new screening methodology and in the alanine scan, there was a noticeable improvement in fluorescence. Also, it seemed that peptide 1 benefited through the replacement of Tyr with the bulky N\text{al}, possibly because of its hydrophobic nature. In peptide 3, it seems that cysteic acid, a more acidic amino acid, is able to replace aspartic acid with significantly improved affinity, but not glutamic acid as the affinity decreased greatly when the two glutamic acids were replaced. Another large improvement resulted from
the replacement of Thr with β-homo-Thr which contains an extra carbon added into the peptide backbone. Beta-amino acids have the advantage of resembling the activity-related structural features of the natural peptide while offering well defined secondary structure due to their extended backbone.¹⁰⁴ There were also several peptides that were on par with the controls and may be advantageous due to their possession of unnatural amino acids (Table 2.14).

**Table 2.14** Sequences of peptides containing unnatural amino acids with comparable values to their respective positive control

<table>
<thead>
<tr>
<th>Original Sequence</th>
<th>FTEAESNMNDLV</th>
<th>VEGEGEEEEEY</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sequences of positive hits</strong></td>
<td>FTeAESNMNDLV</td>
<td>VEGEGeEGeEY</td>
</tr>
<tr>
<td></td>
<td>FTE(Alb)ESNMNDLV</td>
<td>VEGEGeEGeEY</td>
</tr>
<tr>
<td></td>
<td>FTEAESN(Thi)NDLV</td>
<td>VEGEGEeEGeY</td>
</tr>
<tr>
<td></td>
<td>FTEAE(Ser-OMe)NMNDLV</td>
<td>VEGEGEeeGEE(Phe-4-F)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VEGEGE(CA)EGeEY</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VEGEGDDeEGC(A)EY</td>
</tr>
</tbody>
</table>

For each peptide there was an improved affinity seen when the specific position of one amino acid (peptide 3: 4, peptide 1: 8) was changed to its D-amino acid counterpart. There were also comparable affinities with D-amino acid replacements in other positions. It is possible that the combination of two or more D-amino acids may increase the overall affinity. The glutamic acids present in peptide 1 also seem to be more susceptible to other acidic residues (cysteic acid) in comparison to peptide 3 and may potentially benefit from the combination of multiple cysteic acids. Peptide 3 resulted in comparable affinity when the methionine cyclized mimic, Thi, was used as well as when the alcohol functional group of serine was replaced with a methoxy group. Finally, the tyrosine residue in peptide 1 appears as if it can be modified with comparable affinity by similar residues. There were also obvious important amino acids in peptide 3 whose values
significantly decreased when replaced with other amino acids containing different functional groups. Such amino acids include N\textsuperscript{7}, M, L and V. Overall, this screening methodology was successful in identifying initial positive hits with potential improved affinity for RHAMM as well as peptides containing unnatural amino acids with comparable or greater affinity for RHAMM than the controls 1 and 3.

2.5 Conclusions

Synthetic peptides representing the carboxy terminal tail of various tubulin isoforms were discovered to mimic hyaluronan. Through bioinformatics and SPR, the lead candidate peptides were found to target RHAMM with high affinity and specificity. The main goal of this project was to develop the candidate peptides into imaging agents and perform \textit{in vivo} studies. The three peptides were synthesized with a linker and DOTA chelator onto the N-terminus. Afterwards, cold \textsuperscript{69/71}Ga and radioactive \textsuperscript{68}Ga studies were performed for the peptides. Specifically, two of the peptides showed promise and their labelling conditions were optimized to produce good radiochemical yields, and high specific activities and radiopurities. \textit{In vivo} cell studies were performed for the two peptides. The results showed that these peptides are not able to target RHAMM effectively \textit{in vivo}. The second goal of the project was to develop analogues of the peptides incorporating unnatural amino acids to improve \textit{in vivo} stability and potentially improve affinity for RHAMM. First, the critical amino acids were determined by alanine scans and peptide 2 was found not to have any important amino acids, so was no longer used for subsequent studies. For peptides 1 and 3, unnatural bioisosteres were used for the replacement of critical amino acids and amino acids possessing various functional groups were tried for the replacement of noncritical amino acids. A total of 99 peptides were synthesized in small amounts on the tip workstation. The
screening of these peptides consisted of incubating them with RHAMM-coated magnetic/fluorescent beads and analysis using a plate reader. The initial screen resulted in three novel analogues of peptide 3 and six new analogues of peptide 1 with potential improved affinities for RHAMM. In addition, a total of 10 peptides were discovered with comparable affinities for RHAMM, but containing unnatural amino acids. The initial design of the analogues utilized a rational approach only replacing one amino acid at a time to determine specific replacements with improved affinity. Future optimizations may include the combination of several replacements of amino acids that showed improved affinity for RHAMM. This offers the possibility of increased stability in vivo while maintaining the same or better affinity for RHAMM as previously found. As mentioned, this was an initial screen of analogues and the positive hits will need to be synthesized and validated with further studies. Finally, 7 kDa RHAMM was able to be synthesized using SPPS and was shown to maintain its affinity for hyaluronan through an ELISA assay as well as the analogue screen which produced novel sequences. The discovery of the relevancy of the truncated version of RHAMM is extremely valuable for groups working with this protein as it expedites the synthesis and purification procedures, yet results in very high purity products.

2.6 Experimental Procedures

2.6.1 General Experimental procedure for peptides

All standard Fmoc protected amino acids and coupling agents, except HATU (Nova Biochem), were obtained from Peptides International. Unnatural amino acids used in the library were obtained from Chem-Impex, Matrix Scientific and Sigma Aldrich. Fmoc-Rink amide MBHA resin (4-(2',4'-dimethoxyphenyl)-(9-fluorenylmethoxycarbonyl)-aminomethyl)-phenoxy-acetamidonorleucyl-4-methyl benzhydrylamine resin) was
obtained from Nova Biochem. Tentagel S NH\textsubscript{2} resin was obtained from Chem-Impex. RP-C18 Sep-Pak\textsuperscript{c} cartridges were obtained from Waters. \textsuperscript{68}Ge-\textsuperscript{68}Ga generator was obtained from Eckert and Ziegler.

2.6.2 Peptide Syntheses

Fmoc-based solid-phase peptide synthesis was carried out manually or automatically with 0.05 or 0.1 mmol of 0.52 mmol/g Fmoc-Rink amide MBHA resin and a 3-fold excess of the protected amino acids. Fmoc removal, carried out with 20% piperidine in \textit{N,N}-dimethylformamide (DMF) over two cycles (5 and 15 min), rinsing with DMF and dichloromethane (DCM) twice after each cycle and the Kaiser test is performed to confirm the presence of free primary amino groups.\textsuperscript{95} Afterwards, amino acid activation took place with 3 eq HCTU and 6 eq \textit{N,N}diisopropylethylamine (DIPEA) (5 min) and subsequent coupling over 30 and 60 min cycles, rinsing three times with DMF and DCM after each cycle. Coupling of Fmoc-protected aminohexanoic acid (Ahx) and 2-\{2-(2-aminoethoxy)ethoxy\}acetic acid (AEEA) followed the same methodology used in amino acid coupling. FITC coupling was carried out using FITC fluorescent dye (4 eq.) and DIPEA (2 eq.) in DMF for four hours. Biotin was coupled using NHS-biotin (3 eq) and DIPEA (6 eq) in DMF overnight. Full deprotection of synthesized peptides was accomplished using a solution of 95% TFA (v/v), 2.5% H\textsubscript{2}O (v/v), 2.5% triisopropylsilane (v/v) over 5 hours. The cleaved peptides were then precipitated using tert-butyl methyl ether (TBME) and centrifuged at 2200 rpm for 15 min. After decanting, the peptide pellet was rinsed with TBME, vortexed and centrifuged again. Following the removal of the supernatant, the peptide pellets were dissolved in water, frozen at 78°C and lyophilized.

In order to monitor reaction progress and peptide purity via HPLC, small samples of the resin were cleaved using the same procedure as outlined above for full deprotection.
2.6.3 Purification by RP-HPLC / ESI-MS

Peptides were analyzed using a reverse-phase analytical HPLC column (Sunfire™ RP-C18 column 4.6 x 150 mm, 5 μm). This system was equipped with a Waters 600 136 controller, Waters Prep degasser, and Waters MassLynx software (version 4.1). Employed mobile phases were 0.1% TFA in water (eluent A) and 0.1% TFA in CH₃CN (eluent B). The linear gradient used was 10-40% of B with a flow rate of 1.5 mL min⁻¹ over 10 min. The column eluate was monitored using a Waters 2998 Photodiode array detector set at 220, 254 and 400 nm. Peptides were purified using a reverse-phase preparative HPLC column (Sunfire™ Prep RP-C18 OBD™ column 19 x 150 mm, 5 μm) on the same system mentioned above. The detection method along with eluents and gradients were the same as those stated above, with the exception of the flow rate being set at 20 mL min⁻¹. The collected fraction was then lyophilized to a solid and subsequently analyzed by ESI-MS (electrospray ionization mass spectrometry) (Waters Micromass Quattro Micro™ API). Purity of final products was determined by analytical RP-HPLC (220 nm).

2.6.4 ⁶⁹/⁷¹Ga Labeling

In a typical reaction, 5 mg of the DOTA-peptide was dissolved in 0.1 M pH 4 NaOAc/HOAc buffer. Chelation was carried out using a three-fold excess of anhydrous GaCl₃ at 75°C over 30 minutes. Resulting mixture was cooled prior to purification by a plus C18 RP Sep-Pak® (conditioned with 6 mL of ethanol and 25 mL of water). After passing the reaction mixture through the Sep-Pak®, 10 mL of water was used as eluent in order to wash out residual unreacted GaCl₃. A 6 mL aliquot of EtOH was used to wash out the
labelled product. The resulting solution was then dried on a rotary evaporator, mixed with 5 mL of H$_2$O, frozen at -78°C and subsequently lyophilized overnight.

### 2.6.5 $^{68}$Ga Radiolabelling

To a clean glass microwave vessel was added 200 μL of a DOTA-peptide solution (0.1 mg/mL in pH 3.5 HEPES buffer). This aliquot was dissolved in 800 μL of the buffer (10 fold dilution to obtain final solution with μM concentration), to which ~300 MBq $^{68}$Ga was added, freshly eluted from the Ge-68/Ga-68 generator using 3 mL of 0.1 M HCl. Purification and isolation of $^{68}$Ga was achieved using a Phenomenex Strata-X-C 33u polymeric strong cation exchange column with 0.05 N HCl in acetone as the eluent. The reaction mixture was then heated in the reactor of the automated synthesis module at 80°C for 30 minutes (or manually heated in an oil bath at 90°C for 10 minutes), prior to Sep-Pak® purification using a light RP-C18 SPE Sep-Pak cartridge® (conditioned with 5 mL of ethanol and 5 mL of water). After passing the reaction mixture through the Sep-Pak®, 3 mL of water was used as eluent in order to wash out residual unreacted $^{68}$Ga. A 2 mL aliquot of EtOH was used to wash out the radiolabeled product. After evaporation on the V-10 1.6 Biotage® vortex and vacuum evaporation system, the reaction progress and product purity was analyzed using analytical RP-HPLC (Sunfire™ RP-C18 column 4.6 x 150 mm, 5 μm) coupled to a gamma detector, prior to further use in animal studies. HPLC system employed a Waters 1525 Binary HPLC pump, Waters 2487 dual absorbance detector, Waters In-Line degasser and Breeze software (version 3.30).

### 2.6.6 In Vivo Study

*In vivo* imaging studies were conducted in Nod/Scid with tumours either in the fourth mammary gland or flank. The injection of 7c or 9c (3.0-6.0 MBq) was done in the tail
vein of the mice. Mice were euthanized 2 hours post injection. The organs and tumour were then removed and assayed. Biodistribution was calculated as % injected dose/g.

2.6.7 ELISA Binding Assay for RHAMM
A 96-well plate coated with HA (Echelon) was incubated with varying concentrations of biotinylated 7 kDa RHAMM (0-10.5 μM) overnight at 4°C. The next day it was washed three times with 200 μL wash concentrate (K-1205). Streptavidin-alkaline phosphatase (100μL) was added and the plate was incubated at 37°C for 30 min. Wells were washed three times with wash concentrate. Next, 100 μL of p-nitrophenyl phosphate (PNPP) (1 tablet in 11 mL substrate buffer) was added in the dark and absorbance at 405 nm was recorded for each well.

2.6.8 Generation of RHAMM-Coated Magnetic/Fluorescent Screening Beads
Two-micron streptavidin-coated fluorescent (pink) magnetic beads (Spherotech) were washed three times with phosphate buffered saline (PBS) and secured using a strong neodymium magnet to remove sodium azide. 50 μg of the magnetic beads were incubated with 5 μg of biotinylated RHAMM for 1.5 hours at 25°C. 20 μg of biotin was added into the solution and incubated for an additional 30 mins at 25°C to block any remaining streptavidin sites. The RHAMM-coated magnetic/fluorescent beads were washed three times in PBS and secured using a magnet.

2.6.9 Library Screen
The synthesized peptides on tentagel beads were washed twice with ethanol, and three times with binding buffer (25 mM Tris-HCl, 145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 0.02 % Triton-X 100, pH 7.4). First, the positive and negative control peptides were screened against the beads. The controls (~0.5 mg) were suspended in 100 μl of
binding buffer. 5 μl of RHAMM-coated screening beads were added into each tube containing the tentagel beads. This procedure was performed in triplicate. The initial fluorescence values were measured in a plate reader (Synergy H4: ex 560 nm em 590 nm) before incubation to determine the total number of magnetic beads. They were incubated for 1.5 hours at 37°C with constant shaking. After incubation the beads were washed five times with binding buffer. They were then transferred into a 96-well plate and analyzed on a plate reader. The results of the read determined the limits for a negative and positive hit by calculating the ratio of total magnetic beads:bound magnetic beads and the values were normalized. The same procedure was performed on the synthesized analogues and the normalized values were compared to the control values.
Chapter 3: Truncated GLP-1 Imaging Agents for Pancreatic Beta Cell Imaging

3.1 Introduction

Diabetes mellitus is a metabolic disorder that affects millions of people. It has been increasing worldwide and is expected to reach 438 million by 2030. The striking increase in the prevalence of diabetes leads to a significant socioeconomic problem. It is characterized by chronic hyperglycemia due to defective insulin action, secretion or both. Several processes are involved in the development of diabetes including the destruction of insulin-producing pancreatic beta cells, as well as enhanced resistance to insulin action. The lack of insulin results in abnormalities typically observed in carbohydrate, fat and protein metabolism. Consequently, research into diabetes should aim at the preservation and expansion of pancreatic beta cell mass. Molecular imaging techniques can aid in monitoring of the onset of diabetes and its progression into type 2, which may allow for earlier detection and treatment.

Glucagon-like peptide-1 (GLP-1) is a peptide hormone produced by intestinal L-cells in response to nutrient ingestion. It is one of three products formed from the processing reactions of the larger precursor proglucagon. Other peptide fragments include GLP-2 and glicentin. GLP-1 exists predominantly as a 30 amino acid, C-terminally amidated peptide, GLP-1(7-36). It has been shown to reduce glucagon secretion (whose role is to raise blood glucose levels) and strongly increase insulin secretion. GLP-1 exerts its effects by binding to the GLP-1 receptor (GLP-1R). Due to the structure of the receptor, it is classified in the family B subclass of the G protein-coupled receptor (GPCR) superfamily. The structural characteristics of the receptors in this family include a long extracellular N-terminal domain, disulfide bridged cysteine
residues in the extracellular domains, and several glycosylation sites. Structure-function studies of the GLP-1R have shown that the helical region of GLP-1 interacts with the extracellular N-terminal domain of the GLP-1R, while the N-terminal 8 amino acids of GLP-1 interact with residues in the extracellular regions and transmembrane helices of the receptor. The receptor responds to at least four distinct endogenous GLP-1 variants. These include a full-length peptide GLP-1(1-37) and a truncated form GLP-1(7-37), as well as the amidated counterparts GLP-1(1-36)-NH₂ and GLP-1(7-36)-NH₂. The receptor has also been found to respond to the related peptide oxyntomodulin and exogenous mimetic peptides such as exendin-4. Oxyntomodulin is a 37-amino acid peptide found in the colon and binds to both GLP-1R and glucagon receptor, however, its mechanism of action is not known. Exendin-4 is a 39-amino acid hormone found in the saliva of the Gila monster that was first isolated by Dr. John Eng in 1992. It is a very potent and stable GLP-1R agonist with a plasma half-life of 26 min. GLP-1R couples primarily to Gαs, and upon the binding of GLP-1 to its receptor, it activates adenylate cyclase (AC), cAMP production, and an increase in cytosolic calcium that ultimately promotes insulin secretion (Figure 3.1).

Figure 3.1 The role of GLP-1 in insulin secretion
As GLP-1 binds to its receptor on the pancreatic beta cell, it triggers the release of insulin in a glucose-dependent manner which is known as the “incretin effect”. When administered to type 2 diabetic subjects, GLP-1 normalizes blood glucose levels, increases circulating insulin, and diminishes glucagon secretion. GLP-1 receptor signaling events also lead to the stimulation of beta cell proliferation, survival and differentiation.\textsuperscript{114} Thus, GLP-1 may a valuable candidate in the treatment of type 2 diabetes.

The major disadvantage to GLP-1 as a therapeutic agent is its short half-life of 2 min \textit{in vivo}. This is due to the protease, dipeptidyl peptidase-IV (DPP-IV) cleaving the His-Ala dipeptide from the N-terminus.\textsuperscript{114} Through key amino acid substitutions and modifications, discoveries have been made to improve the biological half-life of GLP-1. Examples include the substitution of Ala for D-Ala, Gly or Aib at position eight.\textsuperscript{115} Alanine scanning experiments have shown that amino acids in positions 7, 10, 12, 13 and 15 were directly involved in receptor binding and activation, whereas those in positions 28 and 29 maintained the secondary structure of the peptide necessary for receptor recognition.\textsuperscript{116} It can be concluded from the scan that the N-terminal region is most important for binding to the receptor. Work done by Mapelli and colleagues discovered a minimal sequence GLP-1 analogue that is a potent agonist for GLP-1R.\textsuperscript{117}

The N-terminal 9-mer sequence of GLP-1 was chosen as the truncated version of the peptide because five of the amino acids critical for binding were located in that region. Mapelli and colleagues found that a slightly modified 9-mer peptide sequence of GLP-1 with the addition of two biphenyl alanine residues (BIP 1 & 2) is a nanomolar active full agonist of the GLP-1 receptor (Figure 3.2).\textsuperscript{117}
Figure 3.2 Structures of BIP 1 (23) and BIP 2 (24)\textsuperscript{117}

With a 10-fold increase in activity over the original sequence of GLP-1, the modified truncated version \textbf{26} shows great promise as a therapeutic agent (Table 3.1).

Table 3.1 Comparison of sequences between GLP-1(7-17) and the modified version developed by Mapelli \textit{et al.}\textsuperscript{117}

<table>
<thead>
<tr>
<th>Compd</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>GLP-1(7-17)-amide</td>
<td>H-His-\textbf{Aib}-Glu-Gly-Thr-\textbf{Phe}-Thr-Ser-Asp-\textbf{Val}-Ser-NH$_2$</td>
</tr>
<tr>
<td>26</td>
<td>[\textbf{Aib}$^8$, \textbf{α}-\textbf{Me}-\textbf{Phe}$^{12}$, BIP 2$^{16}$, BIP 1$^{17}$] GLP-1(7-17)-amide</td>
<td>H-His-\textbf{Aib}-Glu-Gly-Thr-\textbf{α-MePhe}-Thr-Ser-Asp-24-23-NH$_2$</td>
</tr>
</tbody>
</table>

We now propose that by creating a radiolabelled analogue of \textbf{26} ([\textbf{Aib}$^8$, \textbf{α}-\textbf{Me}-\textbf{Phe}$^{12}$, BIP 2$^{16}$, BIP 1$^{17}$] GLP-1(7-17)-amide), an imaging probe will be developed for studying pancreatic beta cell mass \textit{in vivo}. This would generate a targeted PET imaging agent for GLP-1R through binding at an allosteric site, which in turn may potentially offer greater receptor selectivity due to higher sequence divergence in comparison to the conserved orthosteric domain.
3.2 Previous Research

In an attempt to image pancreatic islets, our group developed GLP-1(7-36) into an imaging agent and labelled with $^{68}$Ga as well as $^{111}$In. First, an *in vitro* imaging study was carried out using $^{111}$In and the clonal insulin-producing beta cell line INS-1 832/13, which expresses GLP-1R. It demonstrated specific binding of the GLP-1 analogue, as it was displaced by exendin-4. Next, *in vivo* studies were performed, and preliminary data indicated specific uptake of the probe in the pancreas of C57BL/6 mice. Unfortunately, the probe was strongly retained in the kidneys and prevented the visualization of the pancreas in PET and SPECT imaging. Reports have indicated a reason for the high kidney uptake to be the presence of multiple positively charged amino acids in the peptide chain.

One possible solution to this is to use a truncated version of GLP-1(7-36) and greatly reduce the number of charged residues. Modifications have been discovered in order to improve the biological half-life of GLP-1 through key amino acid substitutions. Ala-8 is very susceptible to DPP-IV cleavage, but replacing it with an unnatural amino acid, α-aminoisobutyric acid (Aib), resulted in a more stable analogue while retaining the same potency as native GLP-1. Mapelli and colleagues took a minimal sequence approach that kept the first 9 N-terminal amino acids of GLP-1(7-36). The group modified the sequence and these shortened peptides were assessed *in vitro* for functional activity. The initial scan resulted in an 11 mer peptide containing two biphenylalanine (BIP) residues at the C-terminus. Both BIP residues were found to be critical for activity. The optimized BIP residues contained 2'-ethyl-4'methoxy-BIP (BIP 2) in position 10 and 2'-methyl-BIP (BIP 1) in position 11. A final substitution resulted in a
decrease from EC$_{50} = 7$ nM to 0.28 nM by substituting Phe with α-Me-Phe in position 6 (26).$^{117}$

3.3 Project Goal

The aim of the research is to develop a novel imaging agent based on a DOTA chelated 26 probe that will target GLP-1 receptor. The first step involves the synthesis of the two small molecules, 23 and 24, and afterwards incorporating them into peptides. Next, the EC$_{50}$ and IC$_{50}$ of 26 and its DOTA analogues must be determined, at which point they will be developed into imaging agents by incorporating the radionuclide $^{68}$Ga. This will allow for non-invasive PET imaging which may provide a method of studying pancreatic beta cell mass in vivo and ultimately, the onset and progression of diabetes.

3.4 Results and Discussion

3.4.1 Small Molecule Synthesis

The Fmoc-protected amino acids of BIP 1 and BIP 2 are not commercially available, resulting in the first step of this project being the synthesis of both following a literature procedure.$^{117}$ The two molecules are synthesized following similar synthetic routes, and only differ in the boronic acid used for the Suzuki coupling. The synthesis begins with Boc-L-tyrosine methyl ester which is mesylated to produce 27, requiring no further purification (Scheme 3.1). This compound is the starting material used in the Suzuki coupling for both BIP compounds. For BIP 1, the Suzuki coupling between the triflate 27 with o-toly]boronic acid resulted in the biphenyl product 28 in reasonable yields after flash column chromatography. The reaction needed at least 1.4 equivalents of boronic acid in order to fully react with the starting material. With less, purification became difficult due to the similar R$_f$ values of the starting material and product. The
procedure for the methyl ester deprotection and the Boc deprotection differed from the literature, but both went smoothly with very good yields (29 and 30, respectively). The final step was the Fmoc protection of the amine. The purification was difficult because there was excess Fmoc-OSu after the reaction, which has a similar Rf to the product. In an attempt to eliminate excess Fmoc-OSu for an easier purification, Fmoc-Cl was instead used. The reaction was done in dioxane/water and NaHCO₃ was used as the base with 1.5 eq of Fmoc-Cl. No column was needed as the product precipitated out once dioxane was removed. However, this approach resulted in low yields (<30%) thus Fmoc-OSu was used when repeating this reaction. For this reaction to be successful there needs to be a balance between the amount of remaining starting material and excess Fmoc-OSu after the reaction is complete. Using the conditions shown in scheme 3.1, BIP 1, 23, was produced in a reasonable yield as an off-white solid.

Scheme 3.1 The synthetic route for BIP 1₁₁₇
For the synthesis of BIP 2, the boronic acid was not commercially available and needed to be prepared following a literature method (Scheme 3.2). The first step in the synthesis of (2-ethyl-4-methoxy) phenylboronic acid was reacting 2-ethyl phenol with Mel and base. After refluxing overnight, the reaction was complete but after several attempts with very poor yields, it was suspected that the product 31 is volatile and was being lost on the vacuum pump. The solvent was only removed on the rotary evaporator and yields were calculated based on the solvent remaining that can be seen in the $^1$H NMR. Therefore, an accurate yield can still be obtained by this method and indirectly calculating the yield instead of evaporating off the solvent completely by the vacuum pump and ultimately losing much of the product. The bromination was performed in the dark to ensure it was not a radical reaction initiated by light. The reaction was clean with no need for flash chromatography. The product 32 was dissolved in hexanes and the by-product, succinimide, was insoluble and was filtered off. The final step uses n-BuLi and tri-n-butylborate to produce crude boronic acid. After fully drying the product on vacuum, the solid is stirred in hexanes and the white crystals are filtered off as the pure boronic acid 33. The yield was poor due to much of the boronic acid remaining in the mother liquor and not precipitating. The mother liquor needed to be concentrated several times to produce an adequate yield.
Scheme 3.2 The synthesis of (2-ethyl-4-methoxy) phenylboronic acid\textsuperscript{117}

The synthesis of the Fmoc-protected BIP 2 was similar to the synthesis of BIP 1. The Suzuki coupling was first attempted using the same conditions as BIP 1, but ultimately needed a slightly different procedure in order to go to completion. In reference to the literature, the reaction used four different solvents as well as a higher temperature of 100°C. The triflate 27 coupled with (2-ethyl-4-methoxy) phenylboronic acid 33 resulted in the biphenyl product 34 in reasonable yields after flash column chromatography. Similarly, the boronic acid 33 needed to be at least 1.4 equivalents in order to fully react with the starting material, easing purification. The procedure for the methyl ester deprotection and the Boc deprotection remained the same as with BIP 1 with similar yields (35 and 36, respectively). The final step was the Fmoc protection of the amine, where the purification was difficult because of excess Fmoc-OSu after the reaction, which has a similar $R_f$ to the product. Both DIPEA and NaHCO\textsubscript{3} were tried as bases and Fmoc-Cl was tried instead of Fmoc-OSu, however the conditions giving the best results are shown in scheme 3.3. BIP 2 24 was produced in a reasonable yield as an off-white solid.
Scheme 3.3 The synthetic route for BIP 2^{117}

With the two BIP compounds successfully synthesized and fully characterized, the next step was to include the compounds into the reported 11-mer peptide (26): [Alb^{6}, α-Me-Phe^{12}, BIP 2^{16}, BIP 1^{17}] GLP-1(7-17)-amide.

3.4.2 Peptide Synthesis

The novelty of this project is the development of 26 into an imaging agent. For this to be possible, the peptide must first be synthesized with a lysine on the C-terminus onto which DOTA will be coupled (Figure 3.3). One peptide (37) is directly coupled to the
chelator, while the other (38) contains a mini PEG linker, AEEA (2-[2-(2-aminoethoxy)ethoxy]acetic acid), to add distance between the peptide and the chelator.

![Peptide structures](image)

**Figure 3.3** Structures of [Aib⁸, α-Me-Phe₁², BIP ²¹⁶, BIP ¹¹⁷, Lys¹⁸(DOTA)] GLP-1(7-18)-amide, 37, and [Aib⁸, α-Me-Phe₁², BIP ²¹⁶, BIP ¹¹⁷, AEEA¹⁸, Lys¹⁹(DOTA)] GLP-1(7-19)-amide, 38

The syntheses were done manually and the Kaiser test was used to ensure proper coupling and deprotection took place, particularly for the valuable BIP 1 and BIP 2. Several attempts were performed, varying the coupling times for several of the unnatural amino acids. Particularly, Thr¹¹ proved to be difficult to couple onto α-Me-Phe and needed a more reactive coupling agent, HATU, as well as a longer time period. Once the difficult steps were worked out, the three peptides were synthesized and characterized (Table 3.2).
### Table 3.2 Characterization of peptides by ESI-MS and UHPLC

<table>
<thead>
<tr>
<th>#</th>
<th>Peptide</th>
<th>Calculated</th>
<th>Found</th>
<th>Purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Aib$^8$, α-Me-Phe$^{12}$, BIP 2$^{16}$, BIP 1$^{17}$] GLP-1(7-17)-amide</td>
<td>755.3566</td>
<td>755.3455</td>
<td>&gt;95</td>
</tr>
<tr>
<td>26</td>
<td>[Aib$^9$, α-Me-Phe$^{12}$, BIP 2$^{16}$, BIP 1$^{17}$, Lys$^{18}$(DOTA)] GLP-1(7-18)-amide</td>
<td>1012.9957</td>
<td>1013.0044</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>[M+3H]$^{3+}$ = 675.6643</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>[Aib$^9$, α-Me-Phe$^{12}$, BIP 2$^{16}$, BIP 1$^{17}$, AEEA$^{18}$, Lys$^{19}$(DOTA)] GLP-1(7-19)-amide</td>
<td>1085.5326</td>
<td>1085.5471</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>[M+3H]$^{3+}$ = 724.0244</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>[Aib$^9$, α-Me-Phe$^{12}$, BIP 2$^{16}$, BIP 1$^{17}$, Lys$^{18}$(69/71 Ga-DOTA)] GLP-1(7-18)-amide</td>
<td>1046.4467</td>
<td>1046.4424</td>
<td>94</td>
</tr>
<tr>
<td>37b</td>
<td>[Aib$^9$, α-Me-Phe$^{12}$, BIP 2$^{16}$, BIP 1$^{17}$, Lys$^{18}$(69/71 Ga-DOTA)] GLP-1(7-18)-amide</td>
<td>1118.9836</td>
<td>1118.9816</td>
<td>94</td>
</tr>
<tr>
<td>38b</td>
<td>[Aib$^9$, α-Me-Phe$^{12}$, BIP 2$^{16}$, BIP 1$^{17}$, AEEA$^{18}$, Lys$^{19}$(69/71 Ga-DOTA)] GLP-1(7-19)-amide</td>
<td>1118.9836</td>
<td>1118.9816</td>
<td>94</td>
</tr>
</tbody>
</table>

#### 3.4.3 $^{68}$Ga labeling

In order to confirm that the coordination with gallium is possible in good yields and high specific activity, both naturally occurring 69/71 Ga and radioactive $^{68}$Ga labelled analogues were synthesized for compounds 37 and 38. **Table 3.2** shows the purities of the compounds along with their characterization by ESI-MS. As in previous studies, the peptides were coordinated with cold 69/71 Ga in the presence of a sodium acetate buffer. After the reaction was complete, it was purified using a pre-conditioned Sep Pak C18 plus and characterized using UHPLC-MS (ESI). The structures of each compound and their respective UHPLC trace are shown in **Figure 3.4**.
Figure 3.4 Peptide structures and UPLC traces for A. peptide 38b and B. peptide 37b
The compounds were radiolabelled with $^{68}$Ga using an Eckert and Ziegler $^{68}$Ge/$^{68}$Ga generator connected to a series of synthesis modules. The optimized labelling conditions consisted of using 0.5 M HEPES buffer (pH 3.4) and a manual reaction using 20 µg of precursor and heating for 10 minutes at 90°C (Table 3.3).

**Table 3.3** Optimized labelling conditions for the two truncated GLP-1 analogues

<table>
<thead>
<tr>
<th>Compound number</th>
<th>[Aib$^8$, α-Me-Phe$^{12}$, BIP 2$^{16}$, BIP 1$^{17}$, Lys$^{18}(69/71$Ga-DOTA)] GLP-1(7-18)-amide</th>
<th>[Aib$^8$, α-Me-Phe$^{12}$, BIP 2$^{16}$, BIP 1$^{17}$, AEEA$^{18}$, Lys$^{19}(69/71$Ga-DOTA)] GLP-1(7-19)-amide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor Amount (ug)</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Specific Activity (GBq/µmol)</td>
<td>&gt;30</td>
<td>&gt;4</td>
</tr>
<tr>
<td>Radiochemical yield (%) d.c.</td>
<td>73</td>
<td>4</td>
</tr>
<tr>
<td>Radiopurity (%)</td>
<td>&gt;99</td>
<td>&gt;99</td>
</tr>
</tbody>
</table>

From the results, it is evident both compounds are able to be labelled with $^{68}$Ga in good yields and with high specific activities. The compounds were then tested in *in vitro* studies, to determine the IC$_{50}$, EC$_{50}$ and if they have the potential to be imaging agents for GLP-1R by incubating the radiolabelled compounds with cells that overexpress the GLP-1 receptor.

### 3.4.4 In vitro studies

The first cell-based assay was performed in order to determine the competitive binding affinity for GLP-1R of the control peptide 26 as well as the two candidate peptides, 37 and 38 versus the GLP-1R competitive antagonist exendin-4(9-39). Receptor binding...
was measured by the displacement of $[^{125}\text{I}]-\text{exendin-4(9-39)}$ with increasing concentrations of the $^{69/71}\text{Ga}$-coordinated truncated GLP-1 analogues. However, the peptides proved to show no affinity to the GLP-1R and could not compete with exendin-4(9-39) > 1 μM. The next question posed was if the peptides are able to activate the GLP-1 receptor and stimulate the production of intracellular cAMP. As GLP-1 binds to its receptor on the beta cell, intracellular $\text{G} \alpha_s$ is activated, resulting in the stimulation of transmembrane adenylyl cyclases (tmACs) and an increase of cAMP levels. This in turn promotes glucose-dependent insulin secretion. This is the best studied pathway of GLP-1R, so is the most common test for potential agonists. The peptides were able to increase cAMP concentrations in CHO/GLP-1R cells and show that they can act as GLP-1R agonists (Table 3.4). The peptides were not as potent as the control agonist, exendin-4, but were still able to act as strong agonists for the GLP-1R. Moreover, the addition of the chelator and $^{69/71}\text{Ga}$ did not seem to have much of an influence on the stimulation of cAMP, still obtaining an EC$_{50}$ value in the low nM range.

Table 3.4 EC$_{50}$ values for cold $^{69/71}\text{Ga}$ coordinated GLP-1 analogues and controls

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exendin-4</td>
<td>0.093$^{93\text{cc}}$</td>
</tr>
<tr>
<td>26</td>
<td>8.93</td>
</tr>
<tr>
<td>37b</td>
<td>46</td>
</tr>
<tr>
<td>38b</td>
<td>48</td>
</tr>
</tbody>
</table>

Since the peptides are not able to displace exendin-4(9-39) in a competitive assay which is a potent antagonist of the GLP-1R, but are still able to promote cAMP production, it is thought the peptides are acting as allosteric agonists instead. Allosteric ligands bind at sites distinct from the orthosteric binding site recognized by the receptor’s endogenous
agonist. Allosteric agonists are capable of adjusting the binding of orthosteric ligands. It is possible for the allosteric modulator to act at the same receptor and be an enhancer of one orthosteric ligand, an inhibitor of another and have no effect on a third. The term ago-allosteric modulator has been coined for ligands that display allosteric agonism as well as the ability to modulate the binding of the orthosteric ligand (positively or negatively) instead of just ligands that display agonism or modulation. Within the past decade, interest has grown in targeting ligands to allosteric sites on GPCRs. In order to overcome the limitations of many orthosteric targeted ligands, allosteric ligands were sought to provide novel receptor selectivity. Few allosteric ligands of GLP-1R have been reported, so little is known about the allosteric modulation of GLP-1R. However, of the few compounds that have been reported, there is evidence of an allosteric pocket in the GLP-1 receptor. Some examples of allosteric small molecule modulators for GLP-1R include a substituted quinoxaline, referred to as “compound 2”, a pyrimidine-based compound, BETP, and a substituted cyclobutane, Boc5.

Figure 3.5 Small molecule allosteric modulators of GLP-1R. A. “compound 2” B. BETP C. Boc5

The mechanism of activation and the location of the allosteric pocket of GLP-1R is unknown at this point. In the past, GPCRs have been viewed as monomeric, however, in recent years many studies have shown that GPCRs exist as dimers or high oligomers. Therefore, a “functional receptor” may be a homo- or sometimes even a hetero-dimeric
protein complex, most likely bound to a single hetero-trimeric G protein. It is possible that an allosteric activator may be acting via stimulation of receptor dimerization. In a dimeric system, an allosteric ligand can bind to the “other” receptor protomer within the dimer and thus be able to influence the binding of the endogenous agonist through protein-protein interactions between the two protomers. Others hypothesize the allosteric pocket is likely proximal to or within the transmembrane domains. More research is needed to characterize the allosteric binding site for GLP-1R before such hypotheses can be confirmed. Nevertheless, allosteric ligands offer the prospect of greater receptor selectivity due to higher sequence divergence in allosteric sites across receptor subtypes relative to the conserved orthosteric domain.

Finally, to demonstrate that the truncated GLP-1 analogues are suitable for imaging, an in vitro cell study was done with the $^{68}$Ga labelled peptide 37c with CHO/GLP-1R cells as well as wild type CHO cells that do not express GLP-1 receptor. The study was done in triplicate and approximately 1 million cells were added to each test tube, excluding the background activity tubes which received no cells. Both glass and plastic test tubes were attempted, but it was discovered that glass retained much more background radioactivity than plastic, so the subsequent studies were done in plastic test tubes. In the first several attempts, the blocking experiment was done in advance, incubating the cells with 40 μg of exendin-4 and compound 26, respectively for 1 hour at 37°C. Once the probe was prepared with good specific activity, it was incubated with the cells for 1 hour at 37°C. After washing with buffer several times, the tubes were counted on a gamma counter. However, this method resulted in no significant blocking. It was also observed that the overall process was too long and too many half-lives of $^{68}$Ga were passing. A different approach was used in which the blocking agent (40 μg) is added at the same time as the probe. Also, the time after
incubation was reduced with less washing steps. The results demonstrate that by adding the blocking agent and probe simultaneously to compete for the binding site, it was much more effective than pre-blocking (Figure 3.6).

![Graph showing dose response](image)

**Figure 3.6 In vitro experiment for 37c**

It is evident that there is an interaction with the probe and GLP-1R transfected cells. As was previously discovered, blocking with exendin-4 did not seem to affect the binding since the probe is believed to be an allosteric agonist, binding to a separate site than the orthosteric site. The dose significantly decreased from blocking with 26 which is also expected since 26 should compete with the probe, 37c, for the same binding site. To confirm that the interaction with GLP-1R cells is specific a final cell study was performed. A concentration-dependent blocking study was executed in which radioactive 37c was added to both GLP-1R and CHO cells with increasing concentrations of 26 to block the receptor-specific interaction (Figure 3.7). If there was a specific interaction, there would be a concentration dependent decrease in activity as the concentration of the unlabelled blocking agent increases and replaces the radioactive probe.
It was observed that there is a gradual decrease in activity in the GLP-1R cells, but not in the CHO cells in which the activity remained constant. It can be concluded that the probe is interacting specifically with the GLP-1R cells and are most likely acting at an allosteric site. Therefore, 37c (or 38c) is a promising potential PET agent for imaging beta cell mass in the pancreas which may offer improved selectivity for the GLP-1R and increased in vivo stability from the several unnatural amino acids it contains.

### 3.4 Conclusions

The objective of this project was to synthesize and characterize a truncated GLP-1 analogue and develop it into an imaging agent to target the GLP-1 receptor. This would allow for the non-invasive PET imaging of pancreatic beta cells and monitoring the onset and progression of type 2 diabetes. Before the peptides could be synthesized, the unnatural amino acids BIP 1 and BIP 2 needed to be synthesized since they are not...
commercially available. The synthesis followed a reference procedure with some modifications. After the synthesis and characterization of the small molecules, three peptides were synthesized containing the two BIPs: one with a lysine on the C-terminus, one with a lysine and AEEA linker and the last with no additions (26). The two peptides containing the lysine were developed into imaging agents 37a and 38a incorporating a DOTA chelator onto the lysine side chain. Labelling studies were performed, incorporating both cold $^{68/71}$Ga and radioactive $^{68}$Ga into the two peptides. In both cases, the peptides were labelled with $^{68}$Ga in good radiochemical yields, with high specific activities and excellent radiopurities. To determine their ability to target GLP-1R and act as potential imaging agents, several in vitro binding assays were performed. The first assay determines the affinity of the peptide for GLP-1R and its ability to competitively displace $[^{125}I]$exendin-4(9-39) as a measure of its IC$_{50}$ value. This assay showed that neither peptide was able to displace exendin-4(9-39). However, it was discovered that the peptides were still able to act as GLP-1R agonists and stimulate cAMP production. This result prompted us to hypothesize that the peptides may be acting as allosteric agonists and binding to a site distinct from the site that exendin-4 or GLP-1 targets. Even if the peptides are targeting a different site, they remain potential imaging agents as they do bind to GLP-1R. To determine if the peptides have the potential to image in vivo, an in vitro cell study was performed. The results show that the probe is blocked with the control compound 26 and not with exendin-4. Furthermore, the cell blocking study demonstrates that the probe is binding specifically to the GLP-1R as it showed a concentration dependent decrease in activity with increasing concentrations of 26. In contrast, no significant change in activity was found in CHO cells as the concentration of 26 was increased. This confirms that 37c and/or 38c has potential to be a PET probe for monitoring beta cell mass in the pancreas that may offer greater receptor selectivity in
binding through an allosteric site. In addition, it was previously discovered that the charge distribution of the peptide backbone is important for probe clearance. Compared with GLP-1, the synthesized peptides have a significant decrease in the amount of positive charges which may lead to reduced retention in the kidneys and ultimately better visualization of the probe in the pancreas. However, it is important to note that recent studies have concluded that it is impossible to visualize rodent pancreatic islets \textit{in vivo} and must look at either \textit{ex vivo} organ distribution or larger animal models such as nonhuman primates.\textsuperscript{130}

\section*{3.5 Experimental Procedures}

\subsection*{3.5.1 General Experimental procedure for peptides}

All Fmoc protected amino acids and coupling agents, except Fmoc-Lys(Mtt)-OH (Nova Biochem), HATU (Nova Biochem), and HCTU were obtained from Peptides International. Fmoc-Rink amide MBHA resin (4-\((2',4'\text{-dimethoxyphenyl)-(9-fluorenylmethoxycarbonyl)-aminomethyl)-phenoxy-acetamidonorleucyl-4-methyl benzhydrylamine resin) was obtained from Nova Biochem. RP-C18 Sep-Pak\textsuperscript{©} cartridges were obtained from Waters. \textsuperscript{68}Ge-\textsuperscript{68}Ga generator was obtained from Eckert and Ziegler.

\subsection*{3.5.2 Peptide Syntheses}

Fmoc-based solid-phase peptide synthesis was carried out manually with 0.05 or 0.1 mmol of 0.52 mmol/g Fmoc-Rink amide MBHA resin and a 3-fold excess of the protected amino acids. Fmoc removal, carried out with 20% piperidine in \(N,N\)-dimethylformamide (DMF) over two cycles (5 and 15 min), was followed by amino acid activation with 3 eq O-\((1H-6\text{-Chlorobenzotriazole-1-yl)}-1,1,3,3\text{-tetramethyluronium hexafluorophosphate} (HCTU) and 6 eq \(N,N\) diisopropylethylamine (DIPEA) (5 min) and subsequent coupling
over 30 and 60 min cycles. Coupling of Fmoc-protected 2-[2-(2-aminoethoxy)ethoxy]acetic acid (AEEA) followed the same methodology used in amino acid coupling. Full deprotection of synthesized peptides was accomplished using a solution of 95% TFA (v/v), 2.5% H₂O (v/v), 2.5% triisopropysilane (v/v) over 5 hours. The cleaved peptides were then precipitated using tert-butyl methyl ether (TBME) and centrifuged at 2200 rpm for 15 min. After decanting, the peptide pellet was rinsed with TBME, vortexed and centrifuged again. Following the removal of the supernatant, the peptide pellets were dissolved in water, frozen at 78°C and lyophilized overnight. In order to monitor reaction progress and peptide purity via HPLC, small samples of the resin were cleaved using the same procedure as outlined above for full deprotection.

### 3.5.3 Purification by RP-HPLC / ESI-MS

Peptides were analyzed using a reverse-phase analytical HPLC column (Sunfire™ RP-C18 column 4.6 x 150 mm, 5 μm). This system was equipped with a Waters 600 136 controller, Waters Prep degasser, and Waters MassLynx software (version 4.1). Employed mobile phases were 0.1% TFA in acetonitrile (eluent A) and 0.1% TFA in water (eluent B). The linear gradient used was 30-40% of A with a flow rate of 1.5 mL min⁻¹ over 10 min. The column eluate was monitored using a Waters 2998 Photodiode array detector set at 220 and 254 nm. Peptides were purified using a reverse-phase preparative HPLC column (Sunfire™ Prep RP-C18 OBD™ column 19 x 150 mm, 5 μm) on the same system mentioned above. The detection method along with eluents and gradients were the same as those stated above, with the exception of the flow rate being set at 20 mL min⁻¹. The collected fraction was then lyophilized to a solid and subsequently analyzed by ESI-MS (electrospray ionization mass spectrometry) (Waters Micromass Quattro Micro™ API). Purity of final products was determined by analytical RP-HPLC.
3.5.4 $^{69/71}$Ga Labeling

In a typical reaction, 5 mg of the DOTA-peptide was dissolved in 0.1 M pH 4 NaOAc/HOAc buffer. Chelation was carried out using a three-fold excess of anhydrous GaCl$_3$ at 75°C over 30 minutes. The resulting mixture was cooled prior to purification by a plus C18 RP Sep-Pak$^\circledR$ (conditioned with 6 mL of ethanol and 25 mL of water). After passing the reaction mixture through the Sep-Pak$^\circledR$, 10 mL of water was used as eluent in order to wash out residual unreacted GaCl$_3$. A 6 mL aliquot of EtOH was used to wash out the labelled product. The resulting solution was then dried on a rotary evaporator, mixed with 5 mL of H$_2$O, frozen at -78°C and subsequently lyophilized overnight.

3.5.5 $^{68}$Ga Radiolabeling

To a clean glass microwave vessel was added 200 μL of a DOTA-peptide solution (0.1 mg/mL in pH 3.5 HEPES buffer). This aliquot was dissolved in 800 μL of the buffer to which ~300 MBq $^{68}$Ga was added, freshly eluted from the Ge-68/Ga-68 generator using 3 mL of 0.1 M HCl. Purification and isolation of $^{68}$Ga was achieved using a Phenomenix Strata-X-C polymeric strong cation exchange column (30 mg/mL) with 0.05 N HCl in acetone as the eluent. The reaction mixture was then manually heated in an oil bath at 90°C for 10 minutes, prior to Sep-Pak$^\circledR$ purification using a light RP-C18 SPE Sep-Pak cartridge$^\circledR$ (conditioned with 5 mL of ethanol and 5 mL of water). After passing the reaction mixture through the Sep-Pak$^\circledR$, 3 mL of water was used as eluent in order to wash out residual unreacted $^{68}$Ga materials. A 3 mL aliquot of EtOH was used to wash out the radiolabeled product. After evaporation of the solvent using N$_2$ (g) in an oil bath, the reaction progress and product purity was analyzed using analytical RP-HPLC (Sunfire™ RP-C18 column 4.6 x 150 mm, 5 μm) coupled to a gamma detector, prior to further use in cell studies. HPLC system employed a Waters 1525 Binary HPLC pump,
Waters 2487 dual absorbance detector, Waters In-Line degasser and Breeze software (version 3.30).

### 3.5.6 Binding Assays

All GLP-1 receptor binding studies were conducted using Chinese hamster ovary cells stably transfected with the human GLP-1 receptor (CHO/GLP-1R). Receptor binding was measured by the displacement of $[^{125}\text{I}]-\text{exendin-9-39}$ with increasing concentrations of the synthesized $^{69/71}\text{Ga}$-labeled GLP-1 analogues, using a previously published method. Briefly, CHO/GLP-1R cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen) containing 10% fetal bovine serum (Invitrogen). On the day of the experiment, cells were rinsed 2X with Hank’s buffered saline solution (HBSS) and dissociated in HBSS containing 2 mM EDTA. Approximately one million cells were incubated for 60 min at 37°C in binding buffer (DMEM containing 0.1% Trasylol (Bayer) and 0.1% bovine serum albumin (Sigma), pH 7.4), 0.77 μmol $[^{125}\text{I}]	ext{exendin-4(9-39)}$ (Amersham) and variable concentrations of each GLP-1 analogue ($10^{-6}$ to $10^{-12}$ M). After incubation, cells were centrifuged at 2800 rpm for 15 min. After removal of the supernatants, the cell pellets were washed with 200 μL of cold binding buffer and re-centrifuged before the final pellet was counted in a gamma counter.

### 3.5.7 cAMP Studies

Activation of the GLP-1 receptor by the peptide analogues was assessed by stimulation of cAMP. Briefly, CHO/GLP1R cells were plated at a density of 1x$10^5$ cells/well in a 24-well plate 48 hours prior to the day of the experiment. Cells were incubated for 30 min at 37°C in DMEM containing 1 μM 3- isobutyl-1-methylxanthine (IBMX; Sigma) and increasing concentrations of the peptide analogues ($10^{-6}$ to $10^{-12}$ M). Media was removed, cells were rinsed twice with cold HBSS and scraped in 200 μL of 80% ethanol.
cAMP levels were measured using a cAMP radioimmunoassay kit (Perkin Elmer, Shelton CT) as per the manufacturer’s instructions.

3.5.8 In Vitro Imaging

CHO/GLP-1R cells were added to equal approximately one million cells per plastic tube. Three test tubes containing cells did not receive any radiolabelled peptide; three tubes did not contain any cells and were incubated with 1-2 MBq $^{68}$Ga labelled probe; three tubes with cells were incubated with 1-2 MBq $^{68}$Ga labelled probe; three tubes were incubated with 1-2 MBq $^{68}$Ga labelled probe and 40 µg exendin-4; and three tubes were incubated with 1-2 MBq $^{68}$Ga labelled probe and 40 µg compound 26. The volume of all the test tubes was equal to 500 µL and the tubes were incubated for 1 hour at 37°C. After the incubation period, the tubes were centrifuged and the media decanted off. Cells were rinsed with binding buffer (DMEM + 0.1% BSA [bovine serum albumin]) and centrifuged (4x). The media was decanted off and was counted on a gamma counter. The same procedure was repeated for the concentration-dependent blocking study for both the CHO and GLP-1R cells. Each cell line was blocked with varying concentrations of 26 ($10^{-4}$ to $10^{-9}$ M).

3.5.9 General Experimental Procedure for small molecules

Solvents and reagents were used without further purification, and purchased either from VWR, Fisher Scientific, or Sigma-Aldrich. Sterile, deionized water was used in all aqueous procedures. Analytical TLC was carried out on EMD silica gel 60 F254 plates, and column chromatography was performed using Merck silica gel 60 (230-400 mesh). 1H and 13C NMR data were obtained using a Varian 400 spectrometer. Chemical shifts are reported in parts per million (δ) from tetramethylsilane (0 ppm) and are referenced to
the residual proton in the deuterated solvent: CDCl$_3$ (7.26 ppm) and DMSO-$d_6$ (2.48 ppm) for $^1$H NMR. Multiplicities are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br s (broad singlet). Coupling constants ($J$) are reported in Hertz (Hz). High resolution mass spectra (HRMS) were obtained using electron ionization (EI).

**Synthesis of BIP 1**

**Synthesis of boc-L-tyrosine o-triflate methyl ester, 27**

To a solution of Boc-L-tyrosine methyl ester (10.16 mmol, 3.00 g) and pyridine (25.94 mmol, 2.09 mL) in CH$_2$Cl$_2$ (8 mL), at $-78^\circ$C under N$_2$ was slowly added triflic anhydride (12.19 mmol, 2.05 mL). The solution was stirred for 2 hours. After this time, it was removed from the dry ice, and water was added (12 mL) and the layers were separated. The organic layer was washed with 0.5 M NaOH (2×12 mL) and 15% aqueous citric acid (2×12 mL). The organic layer was dried over MgSO$_4$, filtered and concentrated to give the crude product as a red solid (2.13 g) that required no further purification. Yield: 71%. $^1$H NMR (400MHz, CDCl$_3$) = 7.17-7.24 (m, 4H), 4.99-5.01 (br m, 1H), 4.55-4.58 (br m, 1H), 3.69 (s, 3H), 3.13-3.16 (m, 1H), 3.00-3.03 (m, 1H), 1.38 (s, 9H)

**Synthesis of (S)-methyl 2-(tert-butoxycarbonylamino)-3-(2'-methylbiphenyl-4-yl) propanoate, 28**

Potassium carbonate (3.28 mmol, 399 mg), o-tolylboronic acid (3.51 mmol, 477 mg) and tetrakis(triphenylphosphine) palladium (0.07 mmol, 85 mg) were added to dry toluene (10 mL). The mixture was degassed and flushed with N$_2$ and heated to 80$^\circ$C. At this point, 27 (2.34 mmol, 1.00 g) was dissolved in toluene (1.3 mL) and was maintained at 80$^\circ$C overnight. The following morning, the reaction was cooled and filtered through celite. The filtrate was washed with 0.5 M NaOH (2×5 mL) and 15% citric acid (2×5 mL).
The organic layer was dried over MgSO$_4$, filtered and concentrated. The crude mixture was purified by silica gel column chromatography, eluting with 15% EtOAc in hexanes. The relevant fractions were combined and the solvent reduced by rotary evaporation to yield the pure product as a yellow oil. Yield: 75%.

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.17-7.26 (m, 4H), 4.63-4.67 (br m, 1H), 3.75 (s, 3H), 3.16-3.19 (m, 1H), 3.08-3.11 (m, 1H), 2.27 (s, 3H), 1.42 (s, 9H)

**Synthesis of (S)-2-(tert-butoxycarbonylamino)-3-(2'-methylbiphenyl-4-yl)propanoic acid, 29**

The pure product 28 (1.99 mmol, 733 mg) was dissolved in a 1:1 THF:H$_2$O (6 mL). While stirring at room temperature, 1 M NaOH (4.98 mmol) was added and reacted for 3 hours. After this time, THF was removed during rotary evaporation and EtOAc was added. It was washed twice with 1 M NaHSO$_4$ (2×4 mL). The organic layer was dried over MgSO$_4$, filtered and concentrated to yield the title compound as a pale yellow solid. Yield: 86%.

$^1$H NMR (400 MHz, DMSO-d$_6$): $\delta$ 7.14-7.32 (m, 4H), 4.15-4.18 (br m, 1H), 3.06-3.08 (m, 1H), 2.85-2.89 (m, 1H), 2.21 (s, 3H), 1.32 (s, 9H)

**Synthesis of (S)-1-carboxy-2-(2'-methylbiphenyl-4-yl)ethanaminium trifluoroacetate, 30**

Compound 29 (1.90 mmol, 676 mg) was dissolved in 1:1 DCM:TFA (10 mL) and stirred overnight to form the TFA salt. The next morning, the solution was azeotroped with DCM and CHCl$_3$ and was concentrated under rotary evaporation to yield the salt as a brown solid. Yield: 90%.

$^1$H NMR (400 MHz, DMSO-d$_6$): $\delta$ 7.18-7.32 (m, 4H), 4.15-4.35 (br m, 1H), 3.17-3.19 (m, 1H), 3.12-3.16 (m, 1H), 2.24 (s, 3H)
Synthesis of (S)-2-((9H-fluoren-9-yloxy)carbonylamino)-3-(2’-methylbiphenyl-4-yl)propanoic acid, 23

The TFA salt 30 (1.69 mmol, 627 mg) was dissolved in a 1:4 H₂O:THF (8 mL). DIPEA (10.16 mmol, 1.77 mL) was then added to the solution and once the pH was basic, Fmoc-OSu (2.03 mmol, 685 mg) was added. The reaction stirred at room temperature for 24 hours at which point, THF was removed during rotary evaporation and EtOAc (5 mL) was added. The pH was acidified to pH 2 using 2 M HCl and the layers were separated. The aqueous layer was extracted twice with EtOAc (2×5 mL) and then the organic layers were combined and washed with water (2 mL). The organic layer was dried over MgSO₄, filtered and concentrated. The crude mixture was purified by silica gel column chromatography, eluting with 30% EtOAc in hexanes containing 2% acetic acid. The relevant fractions were combined and the solvent reduced by rotary evaporation to yield 23 as a pure off-white solid.

Yield: 65%. ¹H NMR (400 MHz, DMSO-d₆): δ 7.18-7.32 (m, 4H), 4.55-4.65 (br m, 1H), 3.17-3.19 (m, 1H), 3.12-3.16 (m, 1H), 2.24 (s, 3H) ¹³C NMR (400 MHz, DMSO-d₆): δ 20.1, 36.2, 46.6, 55.4, 65.7, 120.1, 125.2, 125.3, 125.9, 127.0, 127.1, 127.6, 128.8, 129.5, 130.3, 134.7, 136.6, 139.3, 140.7, 141.1, 143.7, 143.8, 156.0, 173.4


Synthesis of (2-ethyl-4-methoxy)phenylboronic acid

Synthesis of 3-ethylanisole, 31

3-Ethylphenol (8.82 mmol, 1.0 mL) and K₂CO₃ (42 mmol, 5.80 g) were added to dry acetone (10 mL) and methyl iodide (42 mmol, 2.6 mL) was added. The mixture was
refluxed overnight. The following morning, the mixture was filtered through a celite pad and was washed with acetone. The filtrate and washes were concentrated, dissolved in DCM, filtered and concentrated to yield 3-ethylanisole 31.

Yield: 55%. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 7.20-7.25 (m, 1H); 6.74-6.84 (m, 3H); 3.83 (s, 3H); 2.65 (q, 2H, J=7.2); 1.28 (t, 3H, J=7.7)

**Synthesis of 4-bromo-3-ethylanisole, 32**

Compound 31 (4.85 mmol, 660 mg) and N-bromosuccinimide (5.34 mmol, 950 mg) in 13 mL acetonitrile stirred in the dark, at room temperature overnight. The mixture was concentrated, redissolved in hexanes and filtered. The product was concentrated to yield 32 as a yellow liquid. Yield: 75%. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 7.35-7.45 (m, 1H); 6.80 (d, 1H, J=2.9 Hz); 6.63 (dd, 1H, J=3.0, 8.7 Hz); 3.80 (s, 3H); 2.74 (q, 2H, J=7.5); 1.23 (t, 3H, J=7.6)

**Synthesis of (2-ethyl-4-methoxy)phenylboronic acid, 33**

A solution of 32 (3.63 mmol, 781 mg) in dry THF (7.5 mL) was cooled to -78°C under N\(_2\). 2.5 M n-BuLi in THF (4.72 mmol, 1.89 mL) was added slowly. Stirring was continued for 2 hours at which point, tri-n-butylborate (5.45 mmol, 1.47 mL) was added at the same temperature. The reaction mixture was allowed to warm to 0°C and was quenched with HCl (8 mL). The layers were separated and the aqueous layer was extracted with EtOAC. The combined organic layers were washed with brine, and the organic layer was dried over MgSO\(_4\), filtered and concentrated. The resulting residue was dried under vacuum for 48 hours and the solid was stirred in hexanes for 1 hour. The pure white solid was collected by vacuum filtration to yield 33.

Yield: 40%. \(^1\)H NMR (400 MHz, DMSO-d\(_6\)): \(\delta\) 7.80 (br s, 2H); 7.41 (d, 1H, J=9);
6.68 (d, 2H, 7.5); 3.70 (s, 3H); 2.75 (q, 2H, J=7.5); 1.14, 3H, J=7.5). \(^{13}\)C NMR (400 MHz, DMSO-d\(_6\)): δ 16.9, 27.9, 54.8, 109.9, 113.8, 135.4, 136.8, 150.6, 160.1.

**Synthesis of BIP 2\(^{117}\)**

**Synthesis of (S)-methyl 2-(tert-butoxycarbonylamino)-3-(2'-ethyl-4’-methoxybiphenyl-4-yl) propanoate, 34**

Compound 27 (3.51 mmol, 1.50 g) in dry toluene (11 mL) was purged in N\(_2\) for 10 min. Potassium carbonate (4.92 mmol, 680 mg) in water (3.76 mL) and 33 (4.92 mmol, 885 mg) were added to the mixture. Next, tetrakis(triphenylphosphine)palladium (0.25 mmol, 283 mg) was added along with THF (7.4 mL) and ethanol (3 mL). The mixture heated to 100°C for 4 hours. The solvents were removed during rotary evaporation and the residue was dissolved in DCM (20 mL). The organic layer was washed with 10% NaOH (aq) (2×20 mL) and 15% citric acid (aq) (2×20 mL). The organic layer was dried over MgSO\(_4\), filtered and concentrated. The crude mixture was purified by silica gel column chromatography, eluting with 15% EtOAc in hexanes. The relevant fractions were combined and the solvent reduced by rotary evaporation to yield the pure product as a yellow oil. Yield: 74%. \(^1\)H NMR (400MHz , CDCl\(_3\)) = δ 7.10-7.30 (m, 5H); 6.86 (d, 1H, J=2.4 Hz); 6.78 (dd, 1H, J=2.5, 8.4Hz); 4.95-5.05 (br m, 1H); 4.60-4.70 (br m, 1H); 3.85 (s, 3H); 3.71 (s, 3H); 3.05-3.20 (br m, 2H); 2.56 (q, 2H, J=7.5 Hz); 1.43 (s, 9H); 1.11 (t, 3H, J=7.5 Hz)

**Synthesis of (S)-2-(tert-butoxycarbonylamino)-3-(2'-ethyl-4’-methoxybiphenyl-4-yl)propanoic acid, 35**

The pure product 34 (2.59 mmol, 1.07 g) was dissolved in a 1:1 THF:H\(_2\)O (10 mL). While stirring at room temperature, 1 M NaOH (6.47 mmol) was added and reacted for 3 hours. After this time, THF was removed during rotary evaporation and EtOAc was added. It was washed twice with 1 M NaHSO\(_4\) (2×10 mL). The organic layer was dried over
MgSO\textsubscript{4} filtered and concentrated to yield the title compound as a pale yellow solid. Yield: 85%. \textsuperscript{1}H NMR (400MHz, DMSO-\textit{d}\textsubscript{6}): \textit{\delta} 7.26-7.29 (m, 2H); 7.15-7.20 (m, 2H); 7.04 (d, 1H, J=8.3 Hz); 6.75-6.87 (m, 3H); 4.10-4.18 (br m, 1H); 3.77 (s, 3H); 3.02-3.05 (m, 1H); 2.70-2.85 (m, 1H); 2.50 (q, 2H, J= 7.5 Hz); 1.32 (s, 9H); 1.02 (t, 3H, J=7.5 Hz)

**Synthesis of (S)-1-carboxy-2-(2'-ethyl-4'-methoxybiphenyl-4-yl)ethanaminium trifluoroacetate, 36**

Compound 35 (2.19 mmol, 873 mg) was dissolved in 1:1 DCM:TFA (16 mL) and stirred overnight to form the TFA salt. The next morning, the solution was azeotroped with DCM and CHCl\textsubscript{3} and was concentrated under rotary evaporation to yield the salt as a brown solid. Yield: 90%. \textsuperscript{1}H NMR (400MHz, DMSO): \textit{\delta} 7.26-7.29 (m, 2H); 7.15-7.20 (m, 2H); 7.04 (d, 1H, J=8.3 Hz); 6.75-6.87 (m, 3H); 4.10-4.18 (br m, 1H); 3.77 (s, 3H); 3.02-3.05 (m, 1H); 2.70-2.85 (m, 1H); 2.50 (q, 2H, J= 7.5 Hz); 1.02 (t, 3H, J=7.5 Hz)

**Synthesis of (S)-2-((9H-Fluoren-9-yloxy)carbonylamino)-3-(2'-ethyl-4'-methoxybiphenyl-4-yl)propanoic acid, 24**

The TFA salt 36 (2.10 mmol, 871 mg) was dissolved in THF (20 mL) and a solution of NaHCO\textsubscript{3} predissolved in water (30 mL) was then added to the solution. Once the pH was basic, Fmoc-OSu (2.52 mmol, 850 mg) was added. The reaction stirred at room temperature for 48 hours at which point, THF was removed during rotary evaporation and water (40 mL) was added. The aqueous layer was washed with Et\textsubscript{2}O and the layers were separated. The aqueous layer was acidified to pH 1 using 2 M HCl and was extracted with EtOAc. The organic layers were combined and washed with water and brine. The organic layer was dried over MgSO\textsubscript{4}, filtered and concentrated. The crude mixture was purified by silica gel column chromatography, eluting with 30% EtOAc in hexanes containing 2% acetic acid. The relevant fractions were combined and the solvent reduced by rotary evaporation to yield BIP 2 as a pure off-white solid.
Yield: 65%. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 7.88 (2H, d, J=8.0 Hz); 7.82 (d, 1H); 7.67 (2H, t, J=7.0 Hz); 7.30 (4H, t, J=8.0 Hz); 7.15 (2H, d, J=7.9); 6.99 (1H, d, J=8.4); 6.84 (1H, d, J=2.4); 6.75, (1H, d); 4.10-4.30 (4H, m); 3.76 (3H, s); 3.10-3.20 (1H, m); 2.88-2.98 (1H, m); 2.43 (2H, q, J=7.6); 0.94 (3H, t, J=7.5). $^{13}$C NMR (400 MHz, DMSO-$d_6$): $\delta$ 14.0, 15.4, 21.1, 25.7, 36.2, 46.6, 55.0, 55.5, 65.7, 111.1, 114.0, 120.1, 125.3, 127.0, 127.6, 128.2, 128.9, 130.7, 133.4, 136.2, 139.2, 140.7, 142.4, 143.7, 143.8, 156.0, 158.6, 173.4

HRMS: m/z [M]$: \text{calc: 521.2202; obs: 521.2210.}$
Chapter 4: Conclusions

Molecular imaging has been shown to play a significant role in modern medicine due to its application in diagnostics and therapeutics. In order for the non-invasive in vivo visualization of biological processes to be feasible, it requires the development of an appropriately designed imaging probe. The research in this dissertation focused on the development and characterization of imaging agents. Both projects use gallium-68, a PET radioisotope that is becoming increasingly prominent in medical imaging. It offers several advantages including its facile production from a generator, low cost and convenient half-life.

The first project involved the development of the previously discovered candidate peptides into imaging agents. To accomplish this, a linker was coupled onto the N-terminus of the peptides and DOTA was synthesized on-resin as part of the solid-phase peptide synthesis. After optimizations of $^{68}$Ga labelling conditions were completed, two peptides 7c and 9c were used in separate in vivo studies in mice containing different RHAMM-expressing cell lines. It was observed that the peptides do not function well as in vivo imaging agents. One reason may be due to the number of natural amino acids present in the sequences that can be degraded by various enzymes which leads to poor stability. The second aspect of this project was to develop novel peptides with affinity to RHAMM as well as improved stability through the incorporation of unnatural amino acids. This work involved the synthesis of 99 peptides on the tip workstation. The parallel synthesis unit allowed the synthesis of many peptides in small screening amounts that would otherwise be impossible to manually synthesize. The screening utilized RHAMM-coated magnetic/fluorescent beads and selected hit peptides based on fluorescence. This methodology provides a versatile and accurate screening strategy.
using bulk magnetic and fluorescence separation. The screening strategy proved to be superior to the more common cell-based method which was inconsistent. Although the screen was consistent for positive and negative results and gave rise to positive hits, the error bars on the controls were large. The methods for washing the beads will need to be optimized in the future to obtain more precise results.

The “beads on a bead” strategy requires the protein of interest to adsorb onto the magnetic/fluorescent beads in order to detect positive hit peptides. Typically, purified recombinant RHAMM is used in assays in which the protein is required, however, its synthesis is a very lengthy procedure that often results in low yields. It was realized that the shortened 7 kDa RHAMM was able to be synthesized as part of solid-phase peptide synthesis. This truncated version of RHAMM has not been previously used for studies, thus its ability to target HA was confirmed by an ELISA assay. This discovery may revolutionize RHAMM studies because the standard technique using recombinant RHAMM is very time consuming and RHAMM is difficult to purify. This method was very quick and can be easily modified without interruption of the binding sites. Thus the protein was completely biotinylated, but did not interfere with the lysines, which is the standard location for biotin. A second confirmation that 7 kDa RHAMM is potent is because the initial screen using this protein resulted in three novel analogues of peptide 3 and six new analogues of peptide 1 with potential improved affinities for RHAMM. One of the replacements with improved affinity was confirmed in the alanine scan, offering validation to the screening method. Many of the improvements that were seen in both peptides were through the replacement of acidic residues with amino acids of similar acidity as well as more acidic, hydrophilic residues, such as cysteic acid. Other improvements included D-amino acid and hydrophobic amino acid replacements. These improvements may have allowed for a change in the conformation of the peptide to allow
for stronger binding to the RHAMM receptor. In addition, a total of 10 peptides were discovered with comparable affinities for RHAMM, but containing unnatural amino acids. This offers the possibility of increased stability in vivo while maintaining the same affinity for RHAMM. The positive hits will need to be synthesized and validated with further studies to confirm their affinity. Afterwards, the validated sequences with improved affinity for RHAMM may be further optimized through the combination of multiple amino acid replacements.

The goal of the second project was to develop a previously discovered peptide, 26, into an imaging agent. In this project, a lysine was added onto the C-terminus and modifications (DOTA) were performed on the side chain of the lysine. It was unknown whether a linker would aid or hinder the peptide’s affinity for GLP-1R, so both versions were synthesized as well as their cold $^{69/71}$Ga analogues. The results of the binding assays demonstrate the peptides are not effective agonists at the orthosteric site, but instead are allosteric agonists binding to a distinct site. They were unable to compete with a potent antagonist of the GLP-1R, exendin-4(9-39) and essentially had no IC$_{50}$ value. However, both peptides seemed to be acting as agonists and managed to stimulate cAMP production to obtain good EC$_{50}$ values. The presence or absence of a linker did not affect the binding affinity so it was not used for further studies due to a shorter synthesis time. The in vitro experiment with the $^{68}$Ga labelled peptide 37c confirmed the results of the IC$_{50}$ through the blocking study as there were no blocking effects seen when attempting to block the binding of the probe to GLP-1R with exendin-4. However, full blocking was observed when self-blocking with 26 was attempted. Although the probe is clearly not binding to the same site as GLP-1 or exendin-4 on the GLP-1 receptor, it is binding somewhere on the receptor in order to produce cAMP. Finally, it was confirmed that the probe was interacting with GLP-1R in a specific manner
through a concentration-dependent blocking study with both GLP-1R and CHO cells. As the concentration of the blocking agent, 26, increased, the activity in the GLP-1R cells gradually decreased demonstrating competitive displacement of the probe. In contrast, the CHO cells exhibited consistently low activity independent of the concentration of 26. Therefore, the peptide(s) 37c and 38c remain promising imaging agents that may target the GLP-1R and provide a non-invasive and direct visualization of pancreatic islets in vivo. Future investigations will include the exploration of the functional cooperativity of the allosteric peptide with other GLP-1R agonists as well as an in vivo mouse study to determine the potential of the probe as an imaging agent.

In conclusion, molecular imaging agents using the radionuclide $^{68}$Ga were developed for both projects. The previously discovered peptides in chapter 2 were found not to be adequate imaging agents in vivo and analogues were discovered using a “beads on a bead” screening approach. The analogues that were designed and synthesized have more promise as imaging agents for RHAMM due to the presence of unnatural amino acids that may increase their in vivo stability. In addition, the synthesis of 7 kDa RHAMM by solid-phase peptide synthesis allows for quick and easy production of the protein. The shortened version of the protein has the potential to revolutionize the methods used to explore RHAMM:ligand interactions. It allows for the characterization of the binding profile of RHAMM interacting with a ligand through NMR or mass spectrometry. In addition, it permits access to a variety of screening methods to discover new ligands for RHAMM, such as the “beads on a bead” strategy used in this dissertation. Chapter 3 highlights the synthesis and development of an allosteric agonist for GLP-1 into an imaging agent. Due to the binding at a distinct site it has the potential for greater receptor selectivity and offers a promising PET imaging probe for the visualization of beta cell mass in the pancreas. Furthermore, most current GLP-1R
imaging agents are highly charged and large peptides which results in their high kidney uptake and retention. The imaging probe that was synthesized and discussed offers a new class of GLP-1R imaging agents that are much lower in molecular weight and less charged. These characteristics may allow for decreased kidney uptake and hence the visualization of the pancreas \textit{in vivo}. The small allosteric peptide represents a unique and novel agent for studying type 2 diabetes as well as a tool for further understanding of the allosteric binding site of GLP-1R.
Chapter 5: References


# Curriculum Vitae

**Name:** Ashley Esarik

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The University of Western Ontario  
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2011-2013 MSc in Organic Chemistry (Molecular Imaging)  
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