FUNCTIONAL GROUP INFLUENCE ON UPTAKE AND TUMOUR TO NON-TARGET RATIOS FOR IODINATED MELANOMA IMAGING AND THERAPY AGENTS

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Abstract

This thesis describes the synthesis, characterization, and biological testing of iodinated probes derived from halogenated triazoles, and the effect of substituents on the clearance and tumour to non-target ratios in murine models of melanoma. Following the preparation of a small library of stannylated candidates biodistribution studies for the corresponding radioiodinated probes were performed in C57Bl/6 mice bearing B16F1 tumours. Among the compounds tested an iodinated triazole containing a piperidine and phenyl moiety, $^{125}\text{I}-\text{7g}$, showed the most promising results with high tumour to liver and eye to liver ratios (27 and 81 respectively) at 24 hours. SPECT/CT imaging was consistent and showed excellent tumour visualization with a very high signal to noise ratio, which was attributed to effective clearance of the 1,4 triazole core. As the most promising candidate $^{125}\text{I}-\text{7g}$ offers a platform from which to develop melanin specific radiopharmaceutical with optimal clearance and tumour to non-target ratios.

Using the results from the SAR study, the lead constructs were the basis for preparing second generation agents using antibody recruiting, bioorthogonal targeting and dendrimer platforms. A small molecule, antibody recruiting agent was synthesized, specific to melanoma, a scaffold previously shown successful with prostate cancer. A one pot click reaction was performed between an azide functionalized with the targeting vector, and an alkyne functionalized with a PEG-dinitrophenyl moiety. The reaction produced an iodinated triazole in 50% yield, which was used as a non-radioactive standard. The corresponding $^{125}\text{I}$ derivative was also synthesized through a one pot click
reaction, and isolated in 36% radiochemical yield with greater than 95% radiochemical purity. Unfortunately biodistribution studies showed limited uptake in the tumour (<1 %ID/g) at 24 hours.

As an alternative to active targeting of melanoma, the BZA core was functionalized with a trans-cyclooctene (TCO) group, which is known to undergo a bioorthogonal click reaction with tetrazines in vivo. The TCO derivative was synthesized in 60% overall yield and administered to C57Bl/6 animal model prior to injection of a previously reported iodinated tetrazine. The data showed higher uptake in the tumour and the eyes, 0.7 and 6%ID/g respectively, when compared to the control group, 0.1 and 0.8 %ID/g respectively. These initial results indicate that the pretargeting approach holds significant promise in developing an effective approach to imaging and treating melanoma using medical isotopes.

In an attempt to improve tumour uptake an aminobenzyl piperidine targeting vector, which was used to prepare compound 7g, was linked to a poly (2,2-bis(hydroxymethyl)-propanoic acid) (PMPA) generation one dendrimer through carbamate linkages. The product was isolated in 28% percent yield, and subsequently deprotected and combined with a stannylated triazole in 27% yield. The stannylated derivative was treated with Na\textsuperscript{125}I and iodogen to give the radiolabelled compound in 19% RCY. Unfortunately biodistribution studies showed limited uptake in the tumour (<1 %ID/g) at 24 hours. Two more functionalized dendrimers were synthesized, however due to time constraints the molecules were not evaluated in vivo.
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Chapter 1: Introduction

1.1 Synopsis

The focus of this thesis is the creation of new molecular imaging (MI) probes for imaging and through changing the isotope employed treating metastatic melanoma. The emphasis of the work reported is the synthesis of new iodine containing synthons to establish a theranostic construct for treating this disease whose impact on society is increasing. The introductory material focuses initially on melanoma with additional information on molecular imaging, targeted radiotherapy and isotopes of iodine to demonstrate the rationale for the research and to provide the background relevant to the experimental approach that was taken.

1.2 Melanoma

Skin cancer is the fifth most common cancer in Canada. With early detection and surgical excision of primary tumours, there is a 90% five-year survival rate. Primary tumours generally present themselves as dark asymmetrical moles, which occur most commonly on the calves or torso. A specific strain of skin cancer, melanoma, begins in melanin expressing melanocyte cells. When a primary melanoma metastasizes, late stage disease has a five year survival rate of 16% despite the emergence of new treatments. Melanoma accounts for 4% of all cases of skin cancer, however it is responsible for 74% of all the skin cancer deaths. Currently, the number of melanoma cases in Canada is on the rise, 1.4% in men and 1% in women per year; a trend thought to be caused by
increased leisure time spent in the sun. In 2014, it is estimated that 76 100 people will be diagnosed with invasive melanoma in the United States, and there will be nearly 10 000 deaths.

Imaging modalities such as MRI, CT and ultrasonography can be used to stage melanoma based on the morphological characteristics of any secondary tumours. Differentiating between benign and malignant sites can be difficult with these imaging modalities and very small lesions can be missed. Positron emission tomography using fluorodeoxyglucose (FDG PET) is used to diagnose and stage malignant melanoma, however this method is less selective than other methods as FDG is taken up in areas of high glucose metabolism, and not solely melanomas. It is also difficult to detect a lesion in tissues that have a high glucose metabolism rate, such as the brain, due to the high background in these areas. Among the lack of specificity, FDG cannot be used to create radiotherapeutics for the treatment of metastatic melanoma, consequently there is a need to develop a superior radiopharmaceutical that can detect the disease at an early stage, when the chances of survival are more likely. The complementary therapeutic radiopharmaceutical can then be used to improve quality of life and life expectancy for metastatic melanoma patients.

1.2.1 Current Melanoma Therapies

There are currently limited options for patients with late stage melanoma that result in an increase in mean free survival rates. High dose Interleukin-2, which promotes the generation of lymphokine-activated killer cells (LAK), can be used as an
immunotherapy for melanoma, with a response rate of 16%.\textsuperscript{3} In 2011, BRAF inhibitors were granted FDA approval for the treatment of malignant melanoma.\textsuperscript{5} BRAF V600E mutations are present in 60\% of melanoma cases, where Vemurafenib can be used to inhibit a kinase specific to the V600E mutation.\textsuperscript{6} A phase II study showed that Vemurafenib was able to increase the median patient survival to 16 months, with an overall response rate of 53\%. Although these results are promising, there is a risk with this therapy for reactivation through a different pathway.\textsuperscript{5}

By using a combination of BRAF and mitogen-activated protein kinase (MEK) inhibitors it is possible to achieve a more robust response than either individual treatment. Phase II trials have been conducted with a BRAF V600 inhibitor, Dabrafenib, in combination with a MEK inhibitor, Trametinib, to try and prevent resistance which usually occurs 6 to 7 months after beginning treatment.\textsuperscript{7} The patients that were treated with BRAF and MEK inhibitors had a median progression-free survival of 9.4 months compared to 5.8 months for the BRAF inhibitor alone. Although these treatments appear to be effective, especially in the short term, there is still room for improvement to overall survival.

Immunotherapy using human monoclonal antibodies is also a potential treatment option for patients with late stage melanoma. Ipilimumab is a human IgG1 monoclonal antibody which restricts anti-tumour immune response through a negative regulator of T-cell responses, Cytotoxic T lymphocyte antigen 4 (CTLA-4).\textsuperscript{8} It was found that as the dose of Ipilimumab increased, so did the response rate.\textsuperscript{9} The most effective dose was
determined to be 10 mg/kg, with a response rate of 11.1% compared to 4.2% and 0% in the groups receiving 3 mg/kg and 0.3 mg/kg. Although the results seem promising, the study showed no significant results in the 10 mg/kg group to overall or progression-free survival. Further studies were conducted with Ipilimumab plus Dacarbazine, an alkylating chemotherapy agent, where the combination treatment was able to improve the median overall survival from 8 months without Ipilimumab to 11 months.

Another available immunotherapy, Lambrolizumab, involves inhibiting the PD-1 pathway using an anti-PD1-antibody, which helps release cytotoxic T-cells into the tumour. The PD-1 pathway negatively regulates the generation of immune cells, by blocking this pathway with an antibody, such that the immune cells can be upregulated relative to the native tumour. The phase I trial with this drug showed an overall response rate of 25% with patients receiving a dose of 2 mg/kg every 3 weeks, this increased to 52% with the patients receiving a higher dose of 10 mg/kg every two weeks. Irrespective of the treatments used many patients with metastatic melanoma do not respond, or they ultimately relapse and there is a need for new approaches to enhance outcomes and reduce morbidity.

1.3 Molecular Imaging

Molecular imaging involves exploiting the interaction of energy with tissue to visualize biological and chemical processes at a cellular level. There are many different types of techniques that can be used for molecular imaging including optical, magnetic resonance (MRI), ultrasound (US), single photon emission computed tomography
(SPECT), and positron emission tomography (PET). To gain extra information about the tissue of interest, many of these imaging modalities rely on the use of imaging probes. These probes are used to create contrast within an image, whereas conventional imaging techniques rely on morphological differences in the tissue of interest.\textsuperscript{12} Although MRI has the highest resolution, when employing a contrast agent, it is less sensitive than other techniques such as nuclear and fluorescence imaging. This is apparent when the quantity of agent required to visualize a biological target or process is compared; MRI requires micro to milligram quantities, whereas PET and SPECT require only nanogram quantities of the probe.\textsuperscript{13}

### 1.3.1 Nuclear Molecular Imaging

Nuclear molecular imaging uses radiolabelled compounds in conjunction with radiation detection scanners to visualize a specific biological tissue or process/receptor without perturbing the equilibrium of the system.\textsuperscript{14} Modern nuclear molecular imaging probes, which are also known as targeted radiopharmaceuticals, are typically made up of three components: the targeting vector, the linker, and the radionuclide (Figure 1). The targeting vector is a construct that is known to bind selectively to the site of interest which results in preferential localization of the probe \textit{in vivo}. The linker portion of the molecule connects the targeting vector with the radionuclide through an appropriate distance that does not impede binding. The linker also provides a site for fine-tuning lipophilicity of the probe which is critical for achieving the desired pharmacokinetics and minimizing off-target binding. The radionuclide allows for visualization of the
distribution of the probe *in vivo* through monitoring its characteristic energy emission. The assumption associated with tracer use is the probes are chemically indistinguishable from their non-radioactive form and are therefore treated the same within the biological system under study.\textsuperscript{15} This is made possible in that tracers are administered in such a small quantity there is negligible perturbation of the system consequently nuclear molecular imaging is able to generate biochemical information of a system in its native state.

![Diagram](image)

**Figure 1:** Representation of the components of a targeted radiopharmaceutical

The two types of nuclear imaging methods used clinically are single photon emission computed tomography (SPECT) and positron emission tomography (PET). A SPECT scanner is comprised of detectors derived from inorganic crystals such as sodium iodide (doped with thallium) which emit light when they interact with gamma rays of the appropriate energy.\textsuperscript{14} These photons are converted into an electrical signal through a position sensitive photomultiplier tube, which in turn is used to determine the location of the origin of the signal. A collimator, which is a lead cylinder, limits the field of view in SPECT, therefore localizing the direction of the emission.\textsuperscript{16} The optimal energy range for
the gamma rays emitted from SPECT radionuclides is approximately 100-300 keV.\(^{14}\) Gamma energies less than 100 keV do not readily escape the subject and cannot be efficiently detected while those above 300 keV are too energetic, and penetrate the collimator reducing the sharpness of images while also increasing the dose to the patient.\(^ {14}\)

PET monitors gamma emissions that arise as a result of the annihilation reaction between a positron and an electron. Positrons are emitted from proton rich radionuclides with the appropriate energy, where following annihilation, two 511 keV gamma rays are emitted 180° with respect to one another.\(^ {16}\) The emitted gamma rays are detected as coincident events that can be traced back to the location of the annihilation process which is used to generate the image. PET offers superior sensitivity over SPECT because there is no need for collimation, however commonly use PET isotopes generally have short half life, and the patient is exposed to a higher radiation dose than with SPECT.\(^ {14}\)

A potential disadvantage of both PET and SPECT for diagnostic imaging is the lack of detailed anatomical (structural) information provided by the scan. This challenge has been overcome by combining nuclear imaging methods with computed tomography (CT) or magnetic resonance imaging (MRI), more recently in the case of PET. The combination of the three dimensional structural information of CT/MRI and the biochemical and functional information derived from nuclear imaging creates a very powerful technique for diagnosing, staging and monitoring disease.\(^ {17}\)
1.4 Radiation Therapy

There are currently three common types of radiation therapy used clinically, external beam, brachytherapy, and radionuclide therapy.\textsuperscript{18} External beam is the most common, and involves an external radiation source that is focused to the area of interest. Brachytherapy is the insertion of a sealed radiation source into the tissue of interest to irradiate a localized area. Radionuclide therapy involves the internal delivery of therapeutic medical isotopes to the tissue of interest. The latter involves a radiolabelled molecule, which can be a small molecule, peptide or antibody, carrying an isotope that emits medium to high LET particles that is designed to localize at the specific tissue of interest. This method is particularly suited to treatment of widely disseminated disease as theoretically the radiation dose to non-target tissue is very low as the compound is taken up selectively in the target.\textsuperscript{18}

The radionuclides used for therapy decay through pathways that release cytotoxic radiation in the form of alpha, beta or auger particles.\textsuperscript{18} Alpha decay is the emission of a monoenergetic helium nucleus which occurs most often in elements heavier than lead ($^{211}\text{At}$, $^{213}\text{Bi}$), and some of the lanthanides.\textsuperscript{19} The helium nucleus is released with energy from 2-10 MeV, enough to damage double stranded DNA beyond repair causing cell death.\textsuperscript{14} Due to the large size of the particle it can only travel 50-100 $\mu$m in the body before interacting with tissue. Alpha particles can cause cell death without internalization as they are very high energy. Beta decay is the emission of an energetic electron which can interact with surrounding tissue causing cell death ($^{131}\text{I}$, $^{89}\text{Sr}$). The energetic electron
can travel 0.2-15 mm, and therefore a larger area of tissue will be affected than with alpha particles. Electron capture is a form of radioactive decay that involves a low energy inner shell electron moving into a proton rich nucleus. This converts a proton into a neutron and results in emission of a gamma ray and/or electron to balance the change in energy of the atom ($^{125}$I, $^{123}$I). The low energy electron travels a short distance (nm) therefore the electron emitter must localize near the cell nucleus to be effective.\textsuperscript{18}

The most successful clinical radionuclide therapy has been the use of iodine-131 for the treatment of thyroid cancer.\textsuperscript{18} Na[$^{131}$I] solutions are injected into the patient since iodide is known to accumulate in the thyroid gland. Iodine-131 has also been used to treat neuroendocrine tumours in the form of metaiodobenzylguanidine, which resembles norepinephrine. Other applications of radioiodine as a therapeutic include radiolabelled monoclonal antibodies such as Brexxar. This radiotherapy agent is used to treat CD20 positive non-hodgkins lymphoma.\textsuperscript{18}

1.5 Melanin

Melanin is a group of proteins synthesized in melanocyte cells from tyrosine residues. Melanin acts as a stable free radical and is responsible for protecting the body from UV light through absorption of photons over a wide range of wavelengths. There are two types of melanin in the body; black or brown eumelanin and red or yellow pheomelanin. Eumelanin consists of dihydroxyindole units that contain carboxylic acid functional groups, which are negatively charged at physiological pH.\textsuperscript{20} Eumelanin is
present in the skin, where pheomelanin is not, and therefore eumelanin is the target for melanoma imaging. Melanin is expressed in more than 90% of melanomas and can be targeted by molecules containing highly basic groups, particularly tertiary amines, which interact electrostatically with the negatively charged carboxylic groups on the pigment. It has also been reported in the literature that the presence of π electrons can help promote strong binding to melanin by enhancing π-interactions with the indole groups.

1.5.1 Melanin Targeted Probes

A number of radiopharmaceuticals have been synthesized and evaluated for their ability to target melanin, most of which are developed around a benzamide core. (Figure 2) The interest in aromatic compounds with basic amines began through the use of phenothiazines as pharmaceuticals. High doses of these medications caused pigmentary retinopathy, which lead to further studies of aromatic compounds interacting with melanin. In an attempt to create a brain imaging agent, the Michelot group discovered that there was uptake of their benzamides in the eyes of pigmented mice, but not in that of the albino mice. This prompted the group to use the iodinated benzamides in melanotic melanoma models, in which N-(2-diethylaminoethyl)-4-iodobenzamide (BZA) was the lead agent.

With respect to iodinated radiopharmaceuticals, BZA was one of the earliest melanin imaging agents. It was shown that 123/125I-BZA can target melanin in vivo achieving 0.79 percent injected dose per gram (% ID/g) at 24 hours in B16 mouse models and a tumour to liver ratio of 4.9 ± 0.6. Preclinical imaging studies on murine
melanoma models in C57Bl6 mice showed good tumour visualization and therefore the novel iodinated compound was advanced to clinical studies.

![Chemical Structure](image)

**Figure 2:** Structure of a benzamide, “A” is an aromatic moiety that can be labelled with iodine, “B” is the tertiary amine in which R and R’ are typically alkyl chains

Clinical studies on $[^{123}\text{I}]$BZA were performed in 110 patients with metastatic melanoma, where SPECT images showed high uptake in the liver, kidneys, brain, salivary glands, lungs and bladder at early timepoints. The study determined that 18-24 hours post injection the images were of high quality and maximum sensitivity (Figure 3). The study showed that a tumour less than 7 mm in diameter could not be effectively imaged using SPECT due to the resolution limits at the time. It was however concluded that $[^{123}\text{I}]$BZA could be used to identify ocular melanomas, shown through the visualization of 18 out of 19 ocular tumours. Overall the study showed a 73% sensitivity and 100% specificity on a per patient basis where for FDG the values are 86% and 94% respectively. Although the results seem promising, the clinical data showed significant non-target binding of BZA in the liver and the kidneys which would complicate its use in radiotherapy.
A recent analogue of BZA (MIP-1145), based on the same benzamide core, showed similar distribution in mouse models as BZA, with a significant increase in tumour uptake (Figure 3). The preclinical data was very encouraging, as it was seen that a single dose of the $[^{131}\text{I}]\text{MIP-1145}$ was able to reduce tumour volume by 79% at 35 days, in comparison to untreated tumours. Multiple doses of the radiotherapeutic resulted in tumour regression in comparison to the starting volume.\(^{27}\) The pre-clinical results supported translation of this probe to stage I clinical trials for patients with late stage malignant melanoma. The study involved intravenous injection of 4.0-6.0 mCi of $^{131}\text{I}$ labelled MIP-1145 to patients with confirmed metastatic melanoma.\(^{28}\) Unfortunately the agent, like BZA, shows non-specific binding in the gastrointestinal tract due to the lipophilic nature of the aryl-iodine core which will limit the maximum tolerated dose.\(^{27}\)

More recently a library of cyclic and polycyclic aromatic compounds were synthesized and tested for their ability to target melanin. As mentioned previously, it has been reported that select aromatic groups drive strong binding to melanin due to π stacking interactions. Out of all of the synthesized derivatives, ICF01012 had the highest uptake in the tumour over time, showing tumour retention out to 8 days post-injection.\(^{29}\) Similar to MIP-1145, this agent suffers from high non-target uptake in the stomach and liver at early time points, which is not ideal for therapy.
One argument against the need for a melanin specific agent is that $^{18}$F-fluorodeoxyglucose (FDG) can be used to image melanoma. FDG is a widely used PET tracer that is taken up in tumour cells that have high glucose metabolism in comparison to normal tissue. However, FDG is non-specific as it can be taken up in any tissue that has high glucose metabolism, including sites of inflammation. Existing melanoma treatments can increase the rate of glycolysis in cells, thereby increasing glucose usage and confounding PET scans. A melanoma specific imaging agent that has low non-specific
binding could be used to effectively monitor a patient’s response to melanoma specific therapies including targeted radiotherapy.

Many imaging agents exist in the literature that target melanin, not all of them use iodine as the radionuclide. One successful $^{18}$F derivative is structurally similar to BZA (Figure 4), demonstrated high tumour uptake, greater than 16 %ID/g, in B16F10 melanoma tumours at 2 hours. The PET study showed low background and high tumour uptake, and the SAR work showed that the position of the fluorine as well as the picolinamide nitrogen can drastically change tumour uptake. Another group synthesized a $^{99m}$Tc derivative of BZA and IBP (Figure 5), and found significant tumour uptake and retention especially with the IBP derivative. The compounds were tested in B16F1 melanoma-bearing C57Bl/6 mice, and at 4 hours post injection the tumour had 3.2 %ID/g, however there was high uptake in the liver, kidneys, intestines, and pancreas. Although many agents in the literature seem to be promising for melanoma imaging, it is beneficial to synthesize an iodine based radiopharmaceutical as it can be used for diagnosis and therapy, which the $^{18}$F and $^{99m}$Tc derivatives cannot.

Figure 4: Structure of the lead agent $^{18}$F-2 (left), PET image at 0.5, 1, and 2 hours post injection (right)
Radioiodine is an attractive medical isotope around which to develop radiopharmaceuticals because of the versatility offered by the availability of different radioactive isotopes. Iodine can be used to create isostructural probes from a single construct for both therapy and imaging by simply changing the nature of the isotope. There are four isotopes of iodine that are readily used in nuclear medicine: $^{123}$I, $^{124}$I, $^{125}$I, and $^{131}$I (Table 1).  

With respect to SPECT imaging, $^{123}$I is an attractive isotope because of the ideal gamma emission energy (151 keV) and convenient half-life ($t_{1/2} = 13$ hour). The latter is long in comparison to many common SPECT and PET isotopes which allows for centralized isotope production and transport and therefore does not require an onsite production source. An additional advantage of $^{123}$I is that it can be produced using an accelerator and therefore it does not suffer the supply shortages that have occurred with nuclear reactor-derived isotopes. One main disadvantage is the high cost of $^{123}$I, consequently $^{125}$I which emits a low energy gamma ray, can be used to develop and optimize new radiolabelling reactions, before preparing the more expensive $^{123}$I analogue.
Another pair of iodine isotopes around which groups are increasingly using to develop new diagnostic and therapeutic radiopharmaceuticals are \(^{124}\text{I}\) and \(^{131}\text{I}\). \(^{124}\text{I}\) is a positron emitter that has a long half-life of 4.1 days, making it possible to develop PET tracers to assess compounds with slow pharmacokinetics such is the case with antibodies. PET is a quantitative imaging technique, therefore \(^{124}\text{I}\) labelled compounds can be used to determine the potential effectiveness and dosimetry associated with the \(^{131}\text{I}\) therapeutic analogues.\(^{36}\) As mentioned previously, \(^{131}\text{I}\) emits energetic beta particles (\(\beta_{\text{max}} = 606\) KeV, \(t_{1/2} = 8.02\) days) that can cause cell death making this isotope ideal for targeted treatment of cancer cells. \(^{131}\text{I}\) also emits an imageable gamma ray which is less widely used today as a diagnostic tool alone in an effort to minimize radiation dose.

Table 1: Physical properties of isotopes of iodine used in nuclear medicine\(^{37}\)

<table>
<thead>
<tr>
<th></th>
<th>(^{123}\text{I})</th>
<th>(^{124}\text{I})</th>
<th>(^{125}\text{I})</th>
<th>(^{131}\text{I})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Emission</strong></td>
<td>(\gamma)</td>
<td>(\beta^+)</td>
<td>(\gamma)</td>
<td>(\beta^-)</td>
</tr>
<tr>
<td><strong>Half-Life</strong></td>
<td>13 hours</td>
<td>4 days</td>
<td>60 days</td>
<td>8 days</td>
</tr>
<tr>
<td><strong>Use</strong></td>
<td>SPECT</td>
<td>PET</td>
<td>Therapy/ Method Development</td>
<td>Therapy</td>
</tr>
</tbody>
</table>
1.6.1 Radioiodination

Another advantage of using radioiodine is the variety of reactions that can be used to incorporate the isotope into compounds. Radioiododethallations were one of the first methods used to radiolabel organic molecules with iodine. This was done through the thallation reaction of Tl(TFA)_3 with the radiolabelling precursor in TFA. The substrate is mixed with no carrier added iodine at temperatures of 50-100°C to generate the desired compound. This method has fallen out of favour as it can result in multiple isomers being formed.\(^{35}\)

Radioiododehalogenation involves exchange of a halogen (bromine/iodine) through nucleophillic attack of radioiodine. This reaction involves high temperatures, 160-200 °C, and produces low specific activity compounds due to the challenge of separating the radioactive compound and the radiolabelling precursor. Direct iodination can be performed if a compound exhibits an activated aromatic ring, for example activated tyrosine residues on proteins.\(^{35}\)

Radioiododestannylation is the most commonly used labeling method for small molecules as it involves very mild temperatures and short reaction times, and results in a high specific activity product. Another major advantage of this technique is the regioselective nature of the reaction, which only produces one product, with the radiolabel in the position originally occupied by the metal. Due to the differences in polarity of the radiolabelling precursor and the product, specifically in small molecules, purification is facile and can be accomplished by HPLC or solid-phase extraction (SPE).
A disadvantage of this technique is the potential toxicity of the tin species, and if these compounds are to be used for biological studies it is essential to remove all the tin byproducts and remaining starting material from the radiolabelled product.

### 1.7 Triazole Appending Agent

The Triazole Appending AGent (TAAG), is a new iodine containing synthon developed in the Valliant group. TAAG was designed to be used in place of the iodoaryl group which is commonly used to prepare radiopharmaceuticals including both molecular imaging and therapy agents. TAAG, when iodinated, clears rapidly via the kidneys and shows very little non-specific binding \textit{in vivo}. A biodistribution of the TAAG acid (Figure 6) shows rapid clearance through the renal system, where by 18 hours p.i. most of the activity has been excreted, and only a small amount remains in the thyroid (1.5 %ID/g) which is due either to deiodination or the presence of trace amounts of free iodide in the product.
The first TAAG conjugate to be synthesized was a derivative of an inhibitor against prostate specific membrane antigen (PSMA), which is a protein over expressed in prostate cancer cells. This compound, when iodinated, showed uptake of 15 %ID/g in LNCaP prostate cancer tumours at 24 hours and low non-specific binding (Figure 7) making it an attractive candidate for designing a melanin targeted imaging probe.
1.8 Thesis Overview

The goal of this project is to build on the TAAG core to develop a new imaging and therapy construct for metastatic melanoma. The steps involved include preparing I-TAAG-DEED derivatives containing pharmacokinetic and binding enhancing prosthetic groups through functionalization the TAAG core. This was done through modification of both the A and B section of the TAAG backbone (Figure 8), and then further functionalization once a lead agent was established. This work is presented in Chapter 2. Chapter 3 describes a combined immune and radiotherapy using a small molecule tagged with a dinitrophenyl functional group. Chapter 4 describes an alternative approach where bioorthogonal chemistry is used to detect melanoma. Chapter 5 demonstrates the synthesis of melanin targeted PMPA dendrimers, and the future work of the project.
Figure 8: Schematic representation of the TAAG constructs
Chapter 2- Functional group influence on clearance and tumour to non-target ratios of melanin targeted TAAG probes

2.1 Introduction

As mentioned previously, melanoma accounts for 4% of all cases of skin cancer; however it is responsible for 80% of all the skin cancer deaths.\textsuperscript{40} There are currently limited treatment options for a patient with malignant melanoma; recently approved treatments such as BRAF inhibitors are only effective on strains of melanoma that have the specific BRAF mutation.\textsuperscript{5} While these drugs appear to be effective especially in the short term, they risk reactivation of the inhibited process, and therefore drug resistance. There is a clear need for a therapeutic agent to treat late stage melanoma, and this can be done through targeted radiotherapy. Melanin is an attractive target as it is present in over 90% of melanoma cases, and can be targeted using small molecules containing a tertiary amine.\textsuperscript{21}

Radioiodine still remains one of most used radionuclides in nuclear medicine because of the versatility of the isotopes. Iodine allows the production of isostructural tools for diagnosis and therapy, simply by changing the isotope used to prepare the agent.\textsuperscript{41,37} It has been shown in the literature that altering the nature of the iodine containing prosthetic group can drastically change the pharmacokinetic properties of a probe.\textsuperscript{42,29} Potential melanin targeted radiotherapy agents have been synthesized in the
past, many of which are developed around the benzamide core. These agents show high tumour uptake, however due to the lipophilicity of the iodophenyl core the probes demonstrate non-specific binding *in vivo*. Changing the prosthetic group from iodophenyl to a heterocycle has the potential to improve the target to non-target uptake of a probe by increasing hydrophilicity and therefore decreasing retention in non-target tissue. By decreasing the non-specific binding it is possible to develop a targeted radiotherapy agent that would decrease radiotoxicity in healthy tissue, and provide maximum dose to the tissue of interest.

The Triazole Appending AGent (TAAG), is a new iodine containing synthon developed in our group. TAAG was designed to be used in place of the iodophenyl group present in many of the clinically available radiopharmaceuticals and as a core for creating new molecular imaging and therapy agents. TAAG, when iodinated, clears rapidly via the kidneys and shows very low non-specific binding *in vivo*. The first TAAG conjugate synthesized in the group was a derivative designed to bind prostate specific membrane antigen (PSMA), which is a protein over expressed in prostate cancer cells. This compound, when iodinated, showed high uptake in prostate cancer tumours and low non-specific binding to non-target tissues making it an attractive candidate for designing a melanin targeted imaging probe.

Due to the success of the iodine containing synthons a series of TAAG derivatives were analyzed for their structure-activity relationship (SAR) with respect to melanin binding, beginning with a more polar BZA analogue (Scheme 1).
2.2 Results and Discussion

2.2.1 Chemistry

Compound 1 was synthesized as previously reported, and compound 2 was synthesized according to the literature procedure. This involved the thermal click reaction of an ethynylstannane with an azide resulting in preferential formation of the 1,4 triazole, and a minor side product of the 1,5 isomer. Non-commercially available targeting vectors were synthesized through nucleophilic substitution of the desired benzyl halide with 4-(N-Boc-amino)piperidine. The primary amines were then deprotected with TFA to give the free amines which were coupled to 1 in methanol at 60°C. The compounds were purified through silica gel chromatography to give the radiolabelling precursors 6b to 6g (Scheme 2). Yields of the products ranged from 7 to 76%.
Characterization of all new products was done using $^1$H and $^{13}$C NMR, HRMS and IR. As a representative example, the $^1$H NMR of compound 6d (Figure 10) showed three peaks in the aromatic region, a singlet from the triazole as well as a multiplet and a triplet corresponding to the protons on the phenyl moiety. The peaks in the aliphatic region correlate to the tributyl tin protons, as well as the piperidine ring. It is apparent from the carbon NMR (Figure 11) that tin and fluorine are present based on the splitting pattern. The carbon that is directly bonded to fluorine is shown at 162.2 ppm with a coupling constant of 245 Hz. The peaks at 115 and 130 ppm are also split from the fluorine, however the magnitude of the coupling constant decreases, as expected, with increasing bond number from the fluorine. The peaks at 10, 27 and 29 ppm are split by tin, which is consistent with the proposed structure. The compound was also analyzed by HMBC, to confirm that the desired isomer was produced. This was confirmed though the HMBC which shows a coupling between $C_b$ and $H_a$ (Figure 9, Figure 12). This would not likely be the case if the 1,5-isomer was present as the coupling would be between $C_c$ and $H_a$. 

Scheme 2: Synthesis of compounds 6b-6g
which was not seen. The mass spectrum is also consistent with the structure showing an 
m/z value of 608.2794 and an isotope pattern consistent with the tin moiety (Figure 13).

![Structure of compound 6d](image)

**Figure 9: Structure of compound 6d**

![NMR spectrum](image)

**Figure 10: $^1$H NMR of compound 6d (600 MHz, CDCl₃)**
Figure 11: $^{13}$C NMR of compound 6d (150 MHz, CDCl$_3$)
Figure 12: HMBC of compound 6d, red box indicates critical coupling
A crystal structure of 7g is shown in Figure 14. The length of the C-I bond in the triazole was 2.061(3) Å as compared to 2.0617(18) and 2.097(9) Å in I-TAAG acid (Figure 6) and p-iodobenzoic acid.\textsuperscript{38,43} All bond lengths and angles were comparable to other reported triazoles.\textsuperscript{38,44}
Figure 14: ORTEP representation (50 % thermal probability ellipsoids) of compound 7g

2.2.2 Radiolabelling

The tin precursors 6b-6g were labelled in the presence of no carrier added $^{123/125}$I iodine using an iododestannylation reaction with iodogen as the oxidant. Compounds 6b-6g were radioiodinated in a biphasic system, using chloroform as the solvent. Reactions were complete within 10 minutes, and the products isolated by HPLC in radiochemical yields of 6-51% and radiochemical purity >95% (Table 2). The variability in yield was likely due to the oxidizing agent, which was very sensitive to the method of solution preparation.
Table 2. Radiolabelling results of $^{125}$I labelled Compounds 7b-7g

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>LogP$_{7.4}$</th>
<th>RCY</th>
</tr>
</thead>
<tbody>
<tr>
<td>7b</td>
<td>3,5-OMe</td>
<td>1.02 ± 0.37</td>
<td>12 ± 6%</td>
</tr>
<tr>
<td>7c</td>
<td>3-F</td>
<td>1.39 ± 0.25</td>
<td>21 ± 15%</td>
</tr>
<tr>
<td>7d</td>
<td>4-F</td>
<td>0.84 ± 0.11</td>
<td>37 ± 13%</td>
</tr>
<tr>
<td>7e</td>
<td>2-F</td>
<td>1.14 ± 0.19</td>
<td>40 ± 1%</td>
</tr>
<tr>
<td>7f</td>
<td>3,5-NO$_2$</td>
<td>1.33 ± 0.27</td>
<td>27 ± 18%</td>
</tr>
<tr>
<td>7g</td>
<td>H</td>
<td>0.70 ± 0.19</td>
<td>52 ± 17%</td>
</tr>
</tbody>
</table>

Compounds 6i-6j were radioiodinated in methanol using iodogen as the oxidant. The solvent was changed due to the solubility of the starting materials. Reactions were complete within 10 minutes, and the products isolated by HPLC in radiochemical yields of 19-33% and radiochemical purity higher than 95% (Table 3).
Table 3. Radiolabelling results of $^{125}$I labelled Compounds 7i-7j

<table>
<thead>
<tr>
<th>Compound</th>
<th>LogP$_{7.4}$</th>
<th>RCY</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{125}$I-7i</td>
<td>-0.12 ± 0.02</td>
<td>25 ± 11%</td>
</tr>
<tr>
<td>$^{123}$I-7j</td>
<td>0.63 ± 0.05</td>
<td>19%</td>
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</tbody>
</table>

2.2.3 Lipophilicity Values

The lipophilicity of the radiolabelled compounds is measured to determine the polarity of a compound. This is useful as the clearance of the compound can be influenced by the polarity, and when designing a library of compounds a correlation may be present between the uptake and the log P value. The lipophilicity values of the TAAG derivatives were determined by the shake flask method at pH 7.4 as a representation of physiological conditions. The results are summarized in Tables 2 and 3. The log P values are in the range of -0.12 to 1.39. These values can be compared with the synthesized standard, BZA, which has a log P value of 1.34.\textsuperscript{45}

2.2.4 Biodistribution

The $^{123/125}$I TAAG derivatives were injected in mice bearing B16F1 melanoma tumours to evaluate their structure activity relationship and pharmacokinetic properties. In the literature it has been shown that benzamides have high tumour uptake and clear most non-target tissues by 24 hours therefore 24 hours post-injection was chosen for determination of the distribution of each compound in various organs, fluids and tissues.\textsuperscript{24}
Compound 7h was chosen as the lead compound for our SAR study due to the success of the iodophenyl derivative (BZA).\textsuperscript{24} Compound 7h showed rapid excretion from non-target tissues however minimal tumour uptake. Our first steps were to modify the structure of the molecule to see how varying the substituents on the tertiary amine and
triazole would affect melanin binding. Compound 7j was synthesized since adding an aromatic moiety in the R’ position should increase binding due to the presence of π electrons.\textsuperscript{22} The biodistribution study of 7j showed a ten-fold increase in tumour uptake compared to 7h, however, the ideal clearance that was seen with 7h was compromised. The non-target uptake was thought to be due to the lipophilicity of 7j, therefore 7i was evaluated, which had an intermediate polarity relative to 7h and 7j. Compound 7h resulted in a tumour uptake of 0.8 %ID/g at 24 hours, while achieving ideal clearance. This result was promising as the tumour to blood ratios for 7h and 7i were similar (19 and 17 respectively), while drastically increasing the tumour uptake.

Compound 7g was synthesized to combine the positive results from both 7i and 7j through having both the piperidine and phenyl rings present in the molecule. According to the biodistribution study of 7g tumour uptake was 2.2 %ID/g at 24 hours which represents the highest value among all tested compounds. The results from this study suggest that the binding, retention and clearance of this class of compounds is not solely dependent on the lipophilicity of the compound, but may be due to the location of the lipophilic group. It is known that hydrophillic probes tend to clear through the renal system, and hydrophobic probes tend to clear primarily through the hepatic system.\textsuperscript{46} The log P values for 7j and 7g are 0.63 and 0.70 respectively; however the clearance of the probes was quite different \textit{in vivo}. The tumour to liver ratios obtained were 0.37 ± 0.07 and 27 ± 8 for 7j and 7g, respectively. It seems that retaining the 1,4-triazole core is necessary for rapid
renal clearance, and that substituting this part of the probe may negatively affect distribution in vivo.

Although 7g showed promising results, higher uptake in the tumour is necessary for use as a radiotherapeutic agent; consequently further derivatizations were explored. Altering the electron density of the phenyl group was initially probed through the use of electron donating and electron withdrawing substituents. The presence of the electron donating groups should increase electron density in the ring and therefore interact more strongly with melanin, a π electron accepter. The first derivative 7b contains two methoxy groups, which are electron donating and had a tumour uptake of 1.3 %ID/g at 24 hours. In the second derivative two electron withdrawing nitro groups were substituted on the phenyl ring to give compound 7f which had a tumour uptake of 1.15 %ID/g at 24 hours. Overall the changes in electron density of the phenyl ring were deleterious to binding relative to the parent compound, 7g, which may be due to steric hindrance preventing strong melanin binding.

The next set of derivatives have a fluorine substituted on the aromatic ring which should help decrease the metabolism of the probe in vivo. Three fluorine derivatives substituted at the ortho (7e), meta (7c), or para (7d) positions on the phenyl ring were tested. Unfortunately, all of the fluorinated compounds showed a lower tumour uptake at 24 hours than the parent compound 7g. Although the 24 hour tumour uptake of 7c and 7d were slightly lower than 7g, they retained the excellent tumour to non-target ratios seen with many of the TAAG constructs. It is interesting to note that the presence of the para
fluorine drastically reduced the deiodination of the compound from 7.7 to 2.2 %ID/g at 24 hours, potentially due to the carbon fluorine bond reducing the metabolism of the probe. These values were compared to a known benzamide $^{125}$I-BZA, where it can be seen that although the TAAG derivatives have a lower tumour uptake there is much less non-specific binding.
<table>
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<tr>
<th>Organs</th>
<th>7d</th>
<th>7c</th>
<th>7b</th>
<th>7a</th>
<th>Compound</th>
<th>7f</th>
<th>7i</th>
<th>7h</th>
<th>7g</th>
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<td>0.30</td>
<td>0.09</td>
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<td>0.31</td>
<td>1.32</td>
<td>0.27</td>
<td>1.89</td>
<td>0.72</td>
<td>1.91</td>
<td>0.26</td>
<td>1.03</td>
<td>0.19</td>
<td>0.77</td>
</tr>
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<td>Urine + Bladder</td>
<td>9.04</td>
<td>1.24</td>
<td>0.48</td>
<td>0.06</td>
<td>0.55</td>
<td>0.50</td>
<td>1.85</td>
<td>0.74</td>
<td>0.84</td>
<td>0.44</td>
<td>0.21</td>
</tr>
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</table>

Table 1: Comparison of the biodistribution data for 125I-TAG derivatives in C57Bl6 mice bearing B16F1 tumours at 24 hours p.i.
Due to the rapid clearance of compound 7h and the high tumour uptake of 7g, both of these compounds were used in an imaging study to validate the results achieved in the biodistribution studies. In both cases there was excellent tumour visualization and the uptake mirrors what is seen in the biodistribution studies at 24h (Figure 16). In addition to the tumour, eyes and thyroid are also visualized due to the presence of melanin and the metabolism of the carbon iodine bond in vivo.

Figure 16: SPECT/CT image of $[^{123}\text{I}]7\text{h}$ (A) and $[^{123}\text{I}]7\text{g}$ (B) at 24h post-injection. Tumours are indicated by the yellow arrows.
2.3 Conclusion

In an attempt to improve the clearance of melanin targeted radiopharmaceuticals, nine new iodinated TAAG derivatives were successfully synthesized. All of these compounds containing a tertiary amine were labelled with $^{123}$I or $^{125}$I and evaluated in vivo through biodistribution as well as SPECT imaging studies. It was proven that the presence of the triazole proton is necessary for renal clearance, and that the lipophilicity of these compounds may not be the only factor dictating clearance. Although all of the probes showed tumour uptake, $^{125}$I-7g showed the highest tumour uptake of 2.2 %ID/g at 24 hours in B16F1 tumour model. The compound, labelled with $^{123}$I, reflected the biodistribution study allowing excellent tumour visualization with a high signal to noise ratio in a SPECT/CT imaging study.

2.4 Experimental Section

$^1$H and $^{13}$C NMR spectra were recorded on a Bruker AV 600 spectrometer. High Resolution Mass Spectra were recorded on a Waters Micromass QTof Global mass spectrometer using electrospray ionization (ESI). IR spectra were obtained on a Biorad FTS-40 FTIR spectrometer. SiliaFlash P60 Silica gel from SiliCycle was used for silica gel chromatography. Toluene and tetrahydrofuran were distilled using a PURE SOLV distillation system. FC-72 was purchased from 3M and (CF$_3$(CF$_2$)$_3$(CH$_2$)$_2$)$_3$SnPh was purchased from Fluorous Technologies Inc. All other reagents were purchased from Sigma-Aldrich and A & K Scientific and used without further purification. High pressure liquid chromatography (HPLC) was performed using a Varian ProStar Model 230.
instrument, fitted with a Varian Pro Star model 330 PDA detector monitoring at 254 nm with a model 230 delivery system. Radiolabelled products were purified and monitored with the Waters 2489 Binary HPLC system connected to a 2998 photodiode array detector (monitoring at 254 nm) and a Bioscan γ detector. The UV and γ detectors are connected in series. The radiolabelled compounds were analyzed using Waters X-bridge C18 (5 µm x 10 x 100 mm) column with a binary solvent gradient (4 mL/min) consisting of 90% eluent A for 3 minutes to 0% eluent A over the next 13 min. Eluent A: water containing 0.1% TFA; Eluent B: ACN containing 0.1% TFA (method A). $^{123}$I was produced by MDS Nordion in Vancouver, British Columbia, Canada, using the $^{124}$Xe(p, 2n) reaction and was delivered as a Na$^{123}$I in 0.1M NaOH solution. $^{125}$I was produced by MNR in Hamilton, Ontario, Canada, and was delivered as a Na$^{125}$I in 0.1M NaOH solution.

**General Procedure 1 for the preparation of protected amines (4).**

To a solution of 4-(N-Boc-amino)piperidine (1 equiv) in DCM, was added benzylhalide (1.2 equiv) and DIPEA (1.5 equiv), the reaction was stirred at room temperature for 24h. The reaction mixture was concentrated and purified through column chromatography on silica with 1:1 hexane/ethyl acetate 1% triethylamine.

*tert-butyl 1-(3,5-dimethoxybenzyl)piperidin-4-ylcarbamate (4b)*

See general procedure 1 (35%). $^1$H NMR(CDCl$_3$, 600 MHz) δ 6.48 (d, 2H, J= 1.80 Hz), 6.35 (t, 1H, J= 2.21 Hz), 4.43 (s, 1H), 3.78 (s, 6H), 3.47(s, 1H) 3.41 (s, 2H), 2.78 (m, 2H), 2.08 (m, 2H), 1.90 (m, 2H), 1.44 (s, 11H). $^{13}$C NMR(CDCl$_3$, 150 MHz) δ 160.71,
155.19, 141.10, 106.83, 98.96, 79.21, 63.11, 55.32, 52.36, 47.77, 32.64, 28.43. HRMS (ESI): m/z calcd for C_{19}H_{30}N_{2}O_{4}H: 351.2284 [M + H]^+; found 351.2278. Decomposition temperature 74-77°C.

tert-butyl 1-(3-fluorobenzyl)piperidin-4-ylcarbamate (4c)

See general procedure 1 (57%). ¹H NMR(CDCl₃, 600 MHz) δ 7.25 (m, 1H), 7.05 (m, 2H), 6.92 (m, 1H), 4.47 (s, 1H), 3.46 (s, 3H), 2.77 (m, 2H), 2.09 (m, 2H), 1.90 (m, 2H), 1.44 (m, 11H). ¹³C NMR(CDCl₃, 150 MHz) δ 163.76, 162.14, 155.20, 141.40, 141.37, 129.61, 124.41, 115.67, 115.53, 113.93, 113.79, 79.22, 62.44, 52.36, 47.74, 32.62, 28.42.

tert-butyl 1-(4-fluorobenzyl)piperidin-4-ylcarbamate (4d)

See general procedure 1 (65%). ¹H NMR(CDCl₃, 600 MHz) δ 7.27 (m, 2H), 6.99 (m, 2H), 4.43 (s, 1H), 3.48 (m, 1H), 3.45 (s, 2H), 2.78 (m, 2H), 2.09 (m, 2H), 1.91 (m, 2H), 1.14 (s, 11H). ¹³C NMR(CDCl₃, 150MHz) δ 162.86, 161.23, 155.20, 141.40, 141.37, 114.98, 79.29, 62.18, 52.24, 47.71, 32.49, 28.42.

tert-butyl 1-(2-fluorobenzyl)piperidin-4-ylcarbamate (4e)

See general procedure 1 (93%). ¹H NMR(CDCl₃, 600 MHz) δ 7.38 (m, 1H), 7.24 (m, 2H), 7.11 (m, 1H), 7.02 (m, 1H), 4.42 (s, 1H), 3.59 (s, 2H), 3.47 (m, 1H), 2.84 (m, 2H), 2.19 (m, 2H), 1.92 (m, 2H), 1.43 (s, 11H). ¹³C NMR(CDCl₃, 150MHz) δ 162.21, 160.58, 155.17, 131.66, 128.93, 123.49, 115.34, 115.20, 79.29, 55.14, 52.05, 47.52, 32.39, 28.42.
tert-butyl 1-(3,5-dinitrobenzyl)piperidin-4-ylcarbamate (4f)

See general procedure 1 (92%). $^1$H NMR(CDCl$_3$, 600 MHz) $\delta$ 8.92 (s, 1H), 8.54 (s, 2H), 4.46 (s, 1H), 3.67 (s, 2H), 3.51 (m, 1H), 2.77 (m, 2H), 2.23 (m, 2H), 1.95 (m, 2H), 1.44 (s, 11H). $^{13}$C NMR(CDCl$_3$, 150 MHz) $\delta$ 155.19, 148.59, 144.26, 128.62, 117.68, 79.43, 61.33, 52.53, 47.48, 32.50, 28.41. HRMS (ESI): $m/z$ calcd for C$_{17}$H$_{24}$N$_4$O$_6$H: 381.1774 [M + H]$^+$; found 381.1770. Decomposition temperature 145-148°C.

**General Procedure 1 for the preparation of free amines (5).**

To a solution of Boc protected amine (1 equiv) in DCM was added TFA (10 equiv) and the reaction was stirred at room temperature overnight. The reaction mixture was concentrated, dissolved in 10 mL of saturated sodium bicarbonate and extracted three times with 10 mL of DCM. The organic layer was dried over Na$_2$SO$_4$ and concentrated to give the desired free amine.

1-(3,5-dimethoxybenzyl)piperidin-4-amine (5b)

See general procedure 2 (93%). $^1$H NMR(CDCl$_3$, 600 MHz) $\delta$ 6.48 (s, 2H), 6.34 (s, 1H), 3.77 (s, 6H), 3.42 (s, 2H), 2.82 (m, 2H), 2.64 (m, 1H), 2.01 (m, 2H), 1.77 (m, 2H), 1.47 (m, 2H), 1.42 (s, 2H), 1.38 (m, 2H). $^{13}$C NMR(CDCl$_3$, 150 MHz) $\delta$ 160.67, 141.25, 106.82, 98.91, 63.13, 55.31, 52.48, 48.78, 36.00.
1-(3-fluorobenzyl)piperidin-4-amine (5c)

See general procedure 2 (35%). $^1$H NMR(CDCl$_3$, 600 MHz) δ 7.24 (m, 1H), 7.05 (m, 2H), 6.91 (m, 1H), 3.46 (s, 2H), 2.79 (m, 2H), 2.66 (m, 1H), 2.01 (m, 2H), 1.78 (m, 2H), 1.59 (s, 2H), 1.38 (m, 2H). $^{13}$C NMR(CDCl$_3$, 150 MHz) δ 163.75, 162.12, 141.54 (J= 7.11 Hz) 129.53 (J= 8.32 Hz), 124.42, 115.63 (J= 21.27 Hz), 113.76 (J= 21.27 Hz), 62.45, 52.44, 48.73, 35.88.

1-(4-Fluorobenzyl)piperidin-4-amine (5d)

See general procedure 2 (41%). $^1$H NMR(CDCl$_3$, 600 MHz) δ 7.26 (m, 2H), 6.98 (m, 2H), 3.44 (s, 2H), 2.79 (m, 2H), 2.66 (m, 1H), 1.99 (m, 2H), 1.77 (m, 4H), 1.38 (m, 2H) . $^{13}$C NMR(CDCl$_3$, 150 MHz) δ 162.74, 161.12, 134.29 (J= 2.44 Hz), 130.49 (J= 7.52 Hz), 114.93 (J= 21.05 Hz), 62.22, 52.32, 48.75, 35.78, 29.70 .

1-(2-Fluorobenzyl)piperidin-4-amine (5e)

See general procedure 2 (39%). $^1$H NMR(CDCl$_3$, 600 MHz) δ 7.35 (td, 1H, J= 1.59, 7.55 Hz), 7.21 (m, 1H), 7.08 (td, 1H, J= 0.91, 7.47 Hz), 7.00 (m, 1H), 3.56 (s, 2H), 2.84 (m, 2H), 2.64 (m, 1H), 2.07 (td, 2H, J= 2.03, 11.65 Hz), 1.78 (m, 2H), 1.71 (s, 2H), 1.39 (m, 2H) . $^{13}$C NMR(CDCl$_3$, 150 MHz) δ 162.20, 160.57, 131.54 (J= 4.08 Hz), 128.63 (J= 8.16 Hz), 124.99 (J= 14.28 Hz), 123.80 (J= 2.55 Hz), 115.17 (J= 22.44 Hz), 55.17, 52.18, 48.65, 35.80.
**1-(3,5-Dinitrobenzyl)piperidin-4-amine (5f)**

See general procedure 2 (89%). $^1$H NMR(CDCl$_3$, 600 MHz) δ 8.93 (t, 1H, J = 2.05 Hz), 8.54 (d, 2H, J = 1.98 Hz), 3.68 (s, 2H), 2.80 (m, 3H), 2.18 (td, 2H, J = 2.12, 11.76 Hz), 1.87 (m, 6H), 1.50 (m, 2H). $^{13}$C NMR(CDCl$_3$, 150 MHz) δ 148.57, 144.25, 128.60, 117.63, 61.33, 52.34, 48.43, 35.00. HRMS (ESI): m/z calcd for C$_{12}$H$_{16}$N$_4$O$_4$H: 281.1250 [M + H]$^+$; found 281.1262.

**General Procedure 3 for the conjugation of free amines to TAAG methyl ester (6).**

To a solution of tin TAAG methyl ester (1) (1 equiv) in methanol (1mL) was added amine (1.2 equiv) and DIPEA (3 equiv), the reaction was heated to 60°C for 3 days. The reaction mixture was concentrated and purified through column chromatography on silica with 20:1 dichloromethane/ methanol. The fractions containing the desired compound were concentrated to give the radiolabelling precursor.

**N-(1-(3,5-dimethoxybenzyl)piperidin-4-yl)-2-(4-(tributylstannyl)-1H-1,2,3-triazol-1-yl)acetamide (6b)**

See general procedure 3 (7%). The product was obtained as an orange solid. $^1$H NMR(CDCl$_3$, 600MHz) δ 7.58 (s, 1H), 6.48 (m, 2H), 6.35 (t, 1H, J = 2.18 Hz), 6.21 (s, 1H), 5.07 (s, 2H), 3.77 (s, 7H), 3.44 (s, 2H), 2.77 (m, 2H), 2.15 (m, 2H), 1.84 (m, 2H), 1.54 (m, 6H), 1.48 (m, 2H), 1.32 (m, 6H), 1.12 (m, 6H), 0.87 (t, 9H, J = 7.33 Hz). $^{13}$C NMR(CDCl$_3$, 150MHz) δ 164.78, 160.78, 145.61, 131.46, 99.31, 62.79, 55.33, 52.69, 51.89, 46.78, 31.45, 28.98 (J = 10.72 Hz), 27.19 (J = 29.06 Hz), 13.65, 10.00 (J = 176.76 Hz).
Hz). IR (KBr disc): \( \nu = 3290.61, 2954.69, 2954.24, 2850.88, 1668.97, 1597.26, 1551.53, 1463.42, 1429.70, 1365.97, 1340.69 \); HRMS (ESI): \( m/z \) calcd for \( C_{30}H_{51}N_{5}O_{3}SnH: 650.3098 \); found 650.3111. Melting point 101-104°C.

\( \text{N-(1-(3-Fluorobenzyl)piperidin-4-yl)-2-(4-(tributylstannyl)-1H-1,2,3-triazol-1-yl)acetamide (6c)} \)

See general procedure 3 (20%). The product was obtained as a white solid. \(^{1}\text{H NMR(CDCl\textsubscript{3}, 600 MHz) \delta 7.58 (s, 1H), 7.23 (m, 1H), 7.02 (m, 2H), 6.91 (td, 1H, } \text{J} = 2.08, 8.69 \text{ Hz), 6.05 (s, 1H), 5.06 (s, 2H), 3.78 (m, 1H), 3.44 (s, 2H), 2.69 (m, 2H), 2.10 (t, 2H, } \text{J} = 10.58 \text{ Hz), 1.82 (m, 2H), 1.54 (m, 6H), 1.40 (m, 2H), 1.32 (m, 6H), 1.13 (m, 6H), 0.88 (t, 9H, } \text{J} = 7.37 \text{ Hz)}. \) \(^{13}\text{C NMR(CDCl\textsubscript{3}, 150 MHz) \delta 164.93, 163.76, 162.14, 145.69, 141.21, 131.42 (J= 36.23 Hz), 129.63 (J= 8.02 Hz), 124.34, 115.54 (J= 21.39 Hz), 113.93 (J= 20.91 Hz), 62.26, 52.74, 51.91, 46.86, 31.69, 28.99 (J= 10.70 Hz), 27.19 (J= 29.63 Hz), 13.65, 10.01 (J= 176.84 Hz). IR (KBr disc): \( \nu = 3279.01, 3092.37, 2954.88, 2926.58, 2871.07, 2851.84, 1653.24, 1551.53, 1485.42, 1453.83, 1293.07, 1254.71. \) HRMS (ESI): \( m/z \) calcd for \( C_{28}H_{46}N_{5}O_{3}SnFH: 608.2792 \); found. \( R_f \) (10:1 Dichloromethane/ Methanol)= 0.48.
N-(1-(4-Fluorobenzyl)piperidin-4-yl)-2-(4-(tributylstannyl)-1H-1,2,3-triazol-1-yl)acetamide (6d)

$^1$H NMR(CDCl$_3$, 600 MHz) δ 7.58 (s, 1H), 7.26 (m, 2H), 6.99 (t, 2H, $J$= 8.82 Hz), 6.26 (s, 1H), 5.08 (s, 2H), 3.81 (m, 1H), 3.51 (s, 2H), 2.79 (m, 2H), 2.16 (s, 2H), 1.85 (m, 2H), 1.54 (m, 6H), 1.49 (m, 2H), 1.32 (m, 6H), 1.13 (m, 6H), 0.87 (t, 9H, $J$= 7.11 Hz); $^{13}$C NMR(CDCl$_3$, 150 MHz) δ 165.03, 145.56, 131.48, 130.82, 115.24 ($J$= 19.36 Hz), 61.74, 52.63, 51.74, 46.65, 31.28, 28.91 ($J$= 10.31 Hz), 27.19 ($J$= 27.26 Hz), 13.85, 10.00 ($J$= 176.80 Hz). IR (KBr disc): $\tilde{\nu}$bar = 3291.84, 2954.91, 2923.83, 2851.97, 1668.54, 1602.94, 1551.25, 1464.46; HRMS (ESI): m/z calcd for C$_{28}$H$_{46}$N$_5$OSnF: 608.2792 [M + H]$^+$; 608.2794 found. R$_f$(10:1 Dichloromethane/ Methanol) = 0.46.

N-(1-(2-Fluorobenzyl)piperidin-4-yl)-2-(4-(tributylstannyl)-1H-1,2,3-triazol-1-yl)acetamide (6e)

See general procedure 3 (32%). The product was obtained as an orange oil. $^1$H NMR(CDCl$_3$, 600 MHz) δ 7.33 (m, 1H), 7.21 (m, 1H), 7.07 (m, 1H), 7.00 (m, 1H), 6.04 (s, 1H), 5.05 (s, 2H), 3.76 (m, 1H), 3.53 (s, 2H), 2.74 (m, 2H), 2.15 (t, 2H, $J$= 10.86 Hz), 1.82 (m, 2H), 1.54 (m, 6H), 1.39 (m, 2H), 1.32 (m, 6H), 1.12 (m, 6H), 0.87 (t, 9H, $J$= 7.39 Hz). $^{13}$C NMR(CDCl$_3$, 150 MHz) δ 164.92, 162.16, 160.53, 145.66, 131.40 ($J$= 36.90 Hz), 128.77 ($J$= 7.64 Hz), 123.85, 115.23 ($J$= 22.21 Hz), 55.10, 52.72, 51.74, 46.85, 31.70, 28.98 ($J$= 10.39), 27.19 ($J$= 29.21 Hz), 13.64, 10.00 ($J$= 178.33 Hz). IR (KBr disc): $\tilde{\nu}$bar = 3279.44, 3094.16, 2926.43, 2871.01, 2851.58, 2798.10, 1652.91, 1556.73,
1488.82, 1455.53, 1430.64, 1367.27, 1341.08, 1320.56, 1286.50. HRMS (ESI): \( m/z \) calcd for \( C_{28}H_{46}N_{5}O\text{SnF} \): 608.2792 \([\text{M + H}]^+\); 608.2783 found. \( R_f \) (10:1 Dichloromethane/Methanol)= 0.56

\( N-(1-(3,5\text{Dinitrobenzyl})\text{piperidin-4-yl})-2-(4-(\text{tributylstannyl})-1H-1,2,3\text{-triazol-1-yl})\text{acetamide} \) (6f)

See general procedure 3 (9%). The product was isolated as an orange solid. \(^1\text{H}\) NMR(CDCl\(_3\), 600 MHz) \( \delta \) 8.92 (s, 1H), 8.51 (s, 2H), 7.57 (s, 1H), 6.09 (m, 1H), 5.06 (s, 2H), 3.82 (m, 1H), 3.65 (s, 2H), 2.70 (m, 2H), 2.23 (t, 2H, \( J = 10.89 \text{ Hz} \)), 1.87 (m, 2H), 1.55 (m, 6H), 1.45 (m, 2H), 1.32 (m, 6H), 1.14 (m, 6H), 0.88 (t, 9H, \( J = 7.34 \text{ Hz} \)). \(^{13}\text{C}\) NMR(CDCl\(_3\), 150 MHz) \( \delta \) 164.98, 148.59, 145.77, 143.98, 131.48, 128.51, 117.70, 61.26, 52.75, 52.09, 46.63, 31.63, 28.99 (\( J = 10.30 \text{ Hz} \)), 27.19 (\( J = 29.37 \text{ Hz} \)), 13.66, 10.02 (\( J = 176.73 \text{ Hz} \)). IR (KBr disc): \( \nu_{\text{bar}} = 3262.64, 2954.46, 2924.75, 2851.55, 1656.28, 1539.39, 1458.89, 1343.62; \) HRMS (ESI): \( m/z \) calcd for \( C_{28}H_{45}N_{5}O\text{SnF} \): 680.2588 \([\text{M + H}]^+\); 680.2592 found.

\( N-(1-\text{Benzylpiperidin-4-yl})-2-(4-(\text{tributylstannyl})-1H-1,2,3\text{-triazol-1-yl})\text{acetamide} \) (6g)

See general procedure 3 (64%). \(^1\text{H}\) NMR (CDCl\(_3\), 600 MHz) \( \delta \) 7.57 (s, 1H), 7.29 (m, 4H), 7.23 (m, 1H), 6.08 (bs, 1H), 5.07 (s, 2H), 3.80 (m, 1H), 3.49 (s, 2H), 2.75 (m, 2H), 2.13 (m, 2H) 1.83 (m, 2H) 1.55 (m, 6H), 1.43 (m, 2H), 1.34 (m, 6H), 1.15 (m, 6H), 0.89 (m, 9H), \(^{13}\text{C}\) NMR (CDCl\(_3\), 151 MHz) \( \delta \) 164.92, 145.66, 131.40, 129.11, 128.28, 127.21, 62.81, 52.72, 51.85, 46.83, 31.56, 28.99, 27.20, 13.66, 10.01; IR (KBr disc): \( \nu_{\text{bar}} = \)
3297.14, 3092.37, 2954.47, 2926.72, 2870.74, 2851.24, 2803.70, 2753.74, 1652.63, 1557.94; HRMS (ESI): m/z calcd for C_{28}H_{47}N_{5}OSnH: 590.2886 [M + H]^+; 590.2877 found.

**N-(2-(Piperidin-1-yl)ethyl)-2-(4-(tributylstannyl)-1H-1,2,3-triazol-1-yl)acetamide (6i)**

See general procedure 3 (76%). $^1$H NMR (CDCl$_3$, 600 MHz) $\delta$ 7.61 (s, 1H), 7.11 (s, 1H), 5.11 (s, 2H), 3.40 (q, 2H, $J$ = 5.88, 11.78 Hz), 2.56 (q, 2H, $J$ = 5.88 Hz), 2.50 (s, 4H), 1.60 (m, 4H), 1.53 (m, 6H), 1.46 (s, 2H), 1.33 (m, 6H), 1.11 (m, 6H, $J$ = 8.23, 26.86 Hz), 0.88 (m, 9H, $J$ = 7.39 Hz); $^{13}$C NMR (CDCl$_3$, 150 MHz) $\delta$ 165.87, 145.22, 131.39 ($J$ = 36.98 Hz), 56.45, 54.10, 52.44, 35.68, 28.98 ($J$ = 10.24 Hz), 27.23 ($J$ = 29.93 Hz), 25.07, 23.70, 13.64, 9.95 ($J$ = 176.43 Hz); IR (KBr disc): $\nu$bar = 3301.11, 2930.95, 2871.00, 2852.58, 1683.81, 1558.52, 1540.13, 1456.62; HRMS (ESI): m/z calcd for C$_{23}$H$_{46}$N$_5$OSn: 528.2729 [M + H]$^+$; 528.2735 found.

**N-(2-Diethylamino-ethyl)-2-{5-phenyl-4-[tris-(3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluoro-octyl)stannanyl]-1,2,3-triazol-1-yl}acetamide (6j)**

See general procedure 3 (7.7%). $^1$H NMR (CDCl$_3$, 600MHz) : $\delta$ 7.49 (m, 3H), 7.28 (m, 2H), 4.92 (s, 2H), 3.36 (q, 2H, $J$ = 5.56, 11.13 Hz), 2.60 (m, 6H), 2.18 (m, 6H), 1.18 (m, 6H), 1.01 (t, 6H, $J$ = 7.11 Hz); $^{13}$C (CDCl$_3$, 150MHz) : $\delta$ 166.23, 146.81, 142.84, 131.21, 130.11, 129.94, 127.94, 51.94, 51.47, 47.55, 37.34, 28.13 ($J_{C,Sn}$ = 23.03 Hz), 11.83, 0.00 ($J_{C,Sn}$ = 188.04 Hz); IR (KBr disc): $\nu$bar = 3286, 2929, 1683 cm$^{-1}$; HRMS (ESI): m/z
calcd for $C_{40}H_{34}N_{5}OF_{39}SnH$: 1462.1250 [M + H]$^+$; found 1462.1219, HPLC Rt = 19.10 min.

**General procedure 4 iododestannylation (7).**

To a solution of tin precursor (1 equiv) in THF (1 mL) was added molecular iodine (1.2 equiv) and the reaction was stirred for 10 minutes. At which point the reaction mixture was quenched with an excess of $Na_{2}S_{2}O_{3}$ saturated solution.

*N-(1-(3,5-Dimethoxybenzyl)piperidin-4-yl)-2-(4-iodo-1H-1,2,3-triazol-1-yl)acetamide (7b)*

HRMS (ESI): $m/z$ calcd for $C_{18}H_{24}N_{5}O_{3}IH$: 486.1002 [M + H]$^+$; found 486.0998.

*N-(1-(3-Fluorobenzyl)piperidin-4-yl)-2-(4-iodo-1H-1,2,3-triazol-1-yl)acetamide (7c)*

HRMS (ESI): $m/z$ calcd for $C_{16}H_{19}N_{5}OIF$: 444.0697 [M + H]$^+$; found 444.0695.

*N-(1-(4-Fluorobenzyl)piperidin-4-yl)-2-(4-iodo-1H-1,2,3-triazol-1-yl)acetamide (7d)*

HRMS (ESI): $m/z$ calcd for $C_{16}H_{19}N_{5}OIFH$: 444.0697 [M + H]$^+$; found 444.0706.

*N-(1-(2-Fluorobenzyl)piperidin-4-yl)-2-(4-iodo-1H-1,2,3-triazol-1-yl)acetamide (7e)*

HRMS (ESI): $m/z$ calcd for $C_{16}H_{19}N_{5}OIFH$: 444.0697 [M + H]$^+$; found 444.0716.

*N-(1-(3,5-Dinitrobenzyl)piperidin-4-yl)-2-(4-iodo-1H-1,2,3-triazol-1-yl)acetamide (7f)*

HRMS (ESI): $m/z$ calcd for $C_{16}H_{18}N_{7}O_{3}IH$: 516.0493 [M + H]$^+$; found 516.0486.
N-(1-Benzylpiperidin-4-yl)-2-(4-iodo-1H-1,2,3-triazol-1-yl)acetamide (7g)

$^{1}$H NMR (600 MHz, MeOD) $\delta$ 8.13 (s, 1H), 7.34 (m, 4H), 7.28 (m, 1H), 5.15 (s, 2H), 3.70 (m, 1H), 3.57 (s, 2H), 2.91 (m, 2H), 2.20 (m, 2H), 1.90 (m, 2H), 1.57 (m, 2H); $^{13}$C NMR (151 MHz, MeOD) $\delta$ 166.70, 133.22, 130.85, 129.41, 128.66, 87.62, 63.83, 53.14, 48.23, 32.07 IR (KBr disc): $\nu$ bar = 3293.21, 2923.96, 2802.39, 2761.36, 1668.07, 1550.63, 1450.36 HRMS (ESI): m/z calcd for C$_{16}$H$_{20}$N$_{5}$OI: 427.0827 [M + H]$^+$; found 427.0819

2-(4-Iodo-1H-1,2,3-triazol-1-yl)-N-(2-(piperidin-1-yl)ethyl)acetamide (7i)

$^{1}$H NMR (600 MHz, MeOD) $\delta$ 8.20 (s, 1H), 5.29 (s, 2H), 3.64 (m, 4H), 3.26 (t, 2H, $J$=5.98 Hz), 2.95 (s, 2H), 1.88 (s, 5H), 1.54 (s, 1H); $^{13}$C NMR (151 MHz, MeOD) $\delta$ 168.99, 133.39, 87.80, 57.56, 54.79, 53.19, 35.53, 24.22, 22.66; IR (KBr disc): $\nu$ bar =3315.39, 2936.26, 1683.74, 1623.27, 1558.99, 1447.07, 1253.28, 1203.27, 1130.44; HRMS (ESI): m/z calcd for C$_{11}$H$_{19}$N$_{5}$OI: 365.0660 [M + H]$^+$; 365.0717 found.

N-(2-Diethylamino-ethyl)-2-(4-iodo-[1,2,3]triazol-1-yl)-acetamide (7j)

$^{1}$H NMR (600 MHz, CDCl$_3$) $\delta$ 7.50 (m, 3H), 7.43 (m, 2H), 7.08 (s, 1H), 5.02 (s, 2H), 3.60 (s, 2H), 3.15 (m, 6H), 1.30 (m, 6H); LRMS (ESI): m/z 421

**Radiolabelling**

To a solution of a tin precursor (6b-6f 100 µg) in chloroform (100 uL) was added iodogen (25 µg) in chloroform (25 µL) and acetic acid (5 µL). The reaction was placed on the
shaker for 10 minutes at which point it was quenched with Na$_2$S$_2$O$_3$ (0.1 M, 100 µL). The aqueous layer of the reaction mixture was then purified by RP-HPLC, the desired peak collected, concentrated, and formulated in PBS for biological studies.

To a precoated iodogen (25 µg) vial was added a solution of a tin precursor (6i-6j 100 µg) in methanol (100 µL) and acetic acid (5 µL). The reaction was placed on the shaker for 10 minutes at which point it was quenched with Na$_2$S$_2$O$_3$ (0.1 M, 100 µL). The reaction mixture was then purified by RP-HPLC, the desired peak collected, concentrated, and formulated in PBS for biological studies.

**Biodistribution Studies**

B16F1 cells derived from mouse melanoma were purchased from ATCC (CRL-6323). Female C57Bl/6 mice ordered from Charles River Laboratory (Senneville, QC, Canada) were injected with 4.5 x 10$^5$ B16F1 cells in DPBS subcutaneously into the flank.

Biodistribution studies were performed on mice at 10 days post inoculation ($n = 5$ for $^{123}$I-$7h$, $n = 4$ for $^{125}$I-$7j$, $n = 3$ for the remaining 7 compounds). Mice were injected with 0.48MBq $^{125}$I-$7h$, 0.098MBq $^{125}$I-$7j$, 0.15MBq $^{125}$I-$7i$, 0.22MBq ($^{125}$I-$7g$ and $^{125}$I-$7f$), 0.11MBq $^{125}$I-$7b$. At 24h post-injection, animals were anesthetized with 3% isoflurane and euthanized by cervical dislocation. Blood, adipose, adrenals, bone, brain, eyes, gall bladder, heart, kidneys, large intestine and caecum (with contents), liver, lungs, skeletal muscle, small intestine (with contents), spleen, stomach (with contents), thyroid/trachea, B16F1 tumour, urine + bladder and tail were collected, weighed and counted in a Perkin Elmer Wizard 1470 Automatic Gamma Counter. Decay correction was used to normalize
organ activity measurements to time of dose preparation for data calculations with respect to injected dose (i.e. ID/g).

**Imaging Studies**

Imaging of $^{123}$I-7h and $^{123}$I-7g was completed using female C57Bl/6 mice bearing B16F1 tumours, 10 days post-inoculation. The mice were administered 200µl of PBS containing $^{123}$I-7h (~24.1MBq) or $^{123}$I-7g (~24.9MBq) via tail vein injection. At 24h post-injection, the whole body activity in the mouse was measured. $^{123}$I-7h had largely cleared so the animal was euthanized prior to imaging to allow for longer acquisition times. Prior to imaging for $^{123}$I-7g, the mouse was anaesthetized with 2.5% isoflurane and maintained under the same conditions for the length of the SPECT and CT scans. SPECT acquisitions were completed for 32 frames (300sec/frame for $^{123}$I-7h and 180sec/frame for $^{123}$I-7g) on a GammaMedica Ideas X-SPECT system (North Ridge, California). CT acquisition consisted of 512 projections acquired over 360° with 75Kvp, 205mA cone beam CT system. Cobra Exxim software (Feldkamp filtered backprojection cone beam reconstruction software) was used to reconstruct the images at a voxel size of 155 microns and a matrix size of 512³. An OS-EM interactive reconstructed method (2 iterations/8 subsets) was used to reconstruct the SPECT data which was fused to the CT data using in house software. AMIDE software was used to analyze the images.
Chapter 3: Synthesis of Antibody Recruiting Small Molecules for Treatment of Metastatic Melanoma

3.1 Introduction

The versatile chemistry of the TAAG construct can be exploited to stimulate immune response as well as deliver ionizing radiation as a means to enhance response to radionuclide therapy. Small molecule stimulated immunotherapy has been reported previously by introducing a dinitrophenyl (DNP) group onto a targeting vector, which upon localization at the site of interest (often a tumour) promotes an immune response.

The human body has endogenous antibodies that recognize nitroarene groups that will bind to the DNP group, which in turn signals the cell for destruction by phagocytes.

The first example in the literature of an antibody-recruiting small molecule (ARM) was targeted at prostate specific membrane antigen (PSMA) which is over expressed on prostate cancer tumours (Figure 17). The functionalized inhibitor was prepared using a copper catalyzed 1,3-dipolar cycloaddition reaction with a PEG-DNP alkyne. A range of PEG spacers (n=4, 6, 8, 12) were tested in vitro for their ability to bind the PSMA receptor as well as recruit anti-DNP antibodies. The results of the in vitro studies showed that a smaller PEG chain resulted in stronger binding of the probe to the receptor, with the PEG4 derivative displaying picomolar affinity. However, in the studies to determine the optimal chain length for antibody recruiting, PEG8 had the most activity toward the anti-DNP antibody. ARM P8 lead to cell killing at 30 nM concentration, using antibody concentrations lower than that found in human serum.
The ability to combine radiotherapy and immunotherapy in one construct may represent a convenient way to enhance efficacy. The dual treatment strategy also has the potential to circumvent resistance mechanisms which hinder the treatment of metastatic melanoma. Building on the success of the ARM-P compounds, a melanin targeted PEG8DNP derivative was synthesized and tested *in vivo*. The melanin specific ARM was labelled with radioiodine to help assess the targeting ability, which can, if successful also be used for radiotherapy. To the best of our knowledge the combination of radiopharmaceutical therapy and immunotherapy in one construct has not been reported previously.

![Figure 17: Schematic representation of antibody recruiting small molecule therapy targeting the PSMA receptor (ABT = Antibody-Binding Terminus)](image)

### 3.2 Results and Discussion

The goal was to prepare a construct that contained a melanoma targeting vector, an iodo-TAAG synthon and a DNP group. To this end the same targeting vector for melanin used in chapter 2, *N, N*-diethylethylene diamine was combined with methyl-2-azidoacetate in methanol at 60°C to give the functionalized targeting vector (10, Scheme 3).
Scheme 3: Synthesis of 10

Compound 9 was synthesized as previously reported\(^{49,51}\) and used together with 10 to undergo a three component, one pot click reaction which combines an azide, alkyne, and an electrophilic iodine species to produce an iodinated triazole. The non-radioactive standard was synthesized by combining 9 with 10 in the presence of copper (I) iodide and N-iodosuccinimide (Scheme 4) at room temperature for 5 hours.\(^{52}\) The standard was purified using silica gel chromatography and isolated in 50% yield.

Scheme 4: Synthesis of the non-radioactive standard 11 using the one-pot three component click reaction
The $^1$H NMR of 11 (Figure 18) showed a peak at 5.12 ppm which integrates to 2H and that is characteristic of the protons between the triazole and the amide linkage. Other key features of the spectrum are the aromatic protons representing the DNP group, as well as the absence of a proton relating to the triazole, which is expected as the triazole was iodinated during the one pot reaction. Since the reaction was performed using a copper catalyst there was only formation of one isomer. The high resolution mass spectrum of 11 was comparable to the theoretical isotope model and was therefore used as the standard for the radiolabelling reaction.

Figure 18: $^1$H NMR (600 MHz,CDCl$_3$) of 11
A one pot click reaction was also used for the radiolabelling reaction\textsuperscript{52}, where the procedure employed sodium iodide and copper chloride as the iodine source and the oxidizing agent respectively (Scheme 5).

Scheme 5: Synthesis of $^{125}$I-TAAG-DEED-DNP using the one-pot three component click reaction

The reaction was heated to 60°C for 90 minutes, at which point it was purified by RP-HPLC, concentrated and reconstituted in PBS for biological studies. The log P of the compound was determined to be -0.13 ± 0.04, by the shake-flask method. The log P is comparable with many of the TAAG derivatives synthesized in chapter two, therefore its clearance route should be similar.

A biodistribution study in C57Bl/6 mice bearing B16F1 tumours was performed using two time points; 4 and 24 hours. Tissues and fluids were excised and counted and the percent injected dose per gram (%ID/g) is shown in Figure 20. Unfortunately there
was minimal uptake in the tumour, 0.7 and 0.06 %ID/g at 4 and 24 hours respectively. At 4 hours the gallbladder and large intestine had the highest uptake values of approximately 25 %ID/g, and at 24 hours the thyroid value was approximately 35 %ID/g which indicates a significant amount of deiodination relative to other TAAG constructs which showed less than 10 %. These results show that clearance is not solely guided by lipophilicity, and may be attributed to the size of the compound, which has a molecular weight of 898, larger than the TAAG derivatives which are around 300 amu.

The high uptake in the thyroid can be attributed to free iodine resulting from the loss of iodine in vivo. Stability studies of 11 were performed in the formulated PBS solution, and the compound was stable at least 19 hours after formulation (Figure 19). This result suggests that the presence of free iodine in vivo is likely attributed to metabolism of 11, and not chemical instability.

Figure 19: Gamma HPLC trace of $^{125}$I-11 after 19 hours in PBS.
It is interesting to note that there is limited eye uptake of 11 (0.6 and 0.4 %ID/g at 4 and 24 hours respectively), which is unusual as melanin is also expressed in the eyes of the C57Bl/6 mice. The large size of the PEG8DNP group may cause the compound to behave differently in vivo than expected, by preventing the targeting vector portion from being able to interact with the target. This may be due to the PEG8DNP causing the compound to be taken up in the gall bladder before it can accumulate in the tumour (Figure 20).

Figure 20: Biodistribution data for $^{125}$I-11 in C57Bl/6 mice.

A potential disadvantage of the one-pot labeling technique is the side product of the reaction, which is the formation of a non-iodinated triazole. In the UV HPLC chromatogram of the crude and purified radiolabelled product, there is a peak that elutes
less than 30 seconds before the product which is believed to be the unlabelled triazole (Figure 21). This would typically be a concern as the unlabelled ligand could compete with the tracer for the target. However since the target of interest is melanin, which cannot be saturated at the amounts of compound injected in the study, the specific activity should not influence uptake.

Subsequent to completing these studies, our group discovered that 1,5-iodotriazoles, unlike the isomers used in chapter 2, are prone to deiodination in vivo. In an analogous study a high affinity PSMA derivative labelled using the 3 component one pot reaction showed similarly high levels of iodine in the thyroid (unpublished data). This suggests that in future the 1,4-isomer should be synthesized and evaluated.
3.3 Conclusion

The synthesis and evaluation of a novel melanin targeted iodinated antibody recruiting small molecule was described. The compound was successfully synthesized using a one pot three-component click reaction. The results of the biodistribution study showed that the compound was taken up in the gall bladder, and there is only minimal uptake in the melanin positive tumour. This is thought to be caused by the instability of the molecule and impact of the size of the DNP group and spacer. In the future a
pre-targeting approach could be employed to achieve the desired uptake for a probe that is used as a radio and immunotherapy agent (see chapter 4 for additional details).

3.4 Experimental

$^1$H and $^{13}$C NMR spectra were recorded on a Bruker AV 600 spectrometer. High Resolution Mass Spectra (HRMS) were recorded on a Waters Micromass QToF Global mass spectrometer using electrospray ionization (ESI). IR spectra were obtained on a Biorad FTS-40 FTIR spectrometer. SiliaFlash P60 Silica gel from SiliCycle was used for silica gel chromatography. Toluene and tetrahydrofuran were distilled using a PURE SOLV distillation system. FC-72 was purchased from 3M and $\text{CF}_3\text{(CF}_2\text{)}_5\text{(CH}_2\text{)}_2\text{SnPh}$ was purchased from Fluorous Technologies Inc. All other reagents were purchased from Sigma-Aldrich and A & K Scientific and used without further purification. High pressure liquid chromatography (HPLC) was performed using a Varian ProStar Model 230 instrument, fitted with a Varian Pro Star model 330 PDA detector monitoring at 254 nm with a model 230 delivery system. Radiolabelled products were purified and monitored with the Waters 2489 Binary HPLC system connected to a 2998 photodiode array detector (monitoring at 254 nm) and a Bioscan $\gamma$ detector. The UV and $\gamma$ detectors are connected in series. The radiolabelled compounds were analyzed using Waters X-bridge C18 (5 $\mu$m x 10 x 100 mm) column with a binary solvent gradient (4 mL/min) consisting of 70% eluent A to 65% over 3 minutes to 40% over the next seven minutes to 0% eluent A over the next 5 minutes. Eluent A: water containing 0.1% TFA; Eluent B: ACN
containing 0.1% TFA (method B). $^{125}$I was obtained from the McMaster Nuclear Reactor in Hamilton, Ontario, Canada, and was delivered as a Na$^{125}$I in 0.1M NaOH solution.

**Synthesis of 2-azido-N-(2-(diethylamino)ethyl)acetamide (10)**

To a solution of methyl-2-azidoacetate (0.312 g, 2.7 mmol) in methanol (3 mL) was added $N,N$-diethylethylene diamine (1.14 mL, 8.1 mmol) and DIPEA (1.45 mL, 8.1 mmol). The reaction was heated to 60°C for 48 hours, at which point the reaction mixture was concentrated and purified using a Biotage SP1 (10 g, DCM: MeOH 0-15%). The product was isolated as a yellow oil (13%, 0.07g). $^1$H NMR (600 MHz, CDCl$_3$) δ 6.95 (s, 1H), 3.95 (s, 2H), 3.32 (q, 2H, $J$ = 5.5 Hz), 2.56 (t, 2H, $J$ = 6.1 Hz), 2.53 (q, 4H, $J$ = 7.3 Hz), 1.02 (t, 6H, $J$ = 7.3 Hz).

**Synthesis of N-(2-(diethylamino)ethyl)-2-(4-(25-((2,4-dinitrophenyl)amino)-2,5,8,11,14,17,20,23-octaoxapentacosyl)-5-iodo-1H-1,2,3-triazol-1-yl)acetamide (11)**

To a vial of copper (I) iodide (0.009g, 0.05 mmol) and N-iodosuccinimide (0.013g, 0.05 mmol) under argon was added 2-azido-N-(2-(diethylamino)ethyl)acetamide (0.030g, 0.05 mmol), N-(2,4-dinitrophenyl)-3,6,9,12,15,18,21,24-octaoxaheptacos-26-yn-1-amine (0.010g, 0.05mmol) and triethylamine (0.007 mL, 0.05 mmol) in 300 µL dry DMF. The reaction was stirred at room temperature for 5 hours at which point it was concentrated by rotary evaporation. The crude reaction mixture was purified with silica gel chromatography (Dichloromethane: Methanol (95:5 to 80:20 0.05% TEA)). The fractions containing only the desired product were concentrated by rotary evaporation to yield compound 11 as a yellow oil (0.023 g, 50%). $R_f$ = 0.27; $^1$H NMR (600 MHz, CDCl$_3$) δ
9.13 (d, 1H, $J = 2.8$ Hz), 8.79 (s, 1H), 8.26 (d, 1H, $J = 9.6$ Hz), 6.96 (dd, 1H, $J = 2.8, 9.6$ Hz), 6.82 (s, 1H), 5.12 (s, 2H), 4.63 (s, 2H), 3.83 (t, 2H, $J = 5.4$ Hz), 3.65 (m, 33H), 3.36 (q, 2H, $J = 5.4$ Hz), 2.62 (t, 2H, $J = 5.3$ Hz), 2.57 (q, 4H, $J = 6.9$ Hz), 1.00 (t, 6H, $J = 6.9$ Hz); $^{13}$C NMR (150 MHz, CDCl$_3$) $\delta$ 164.4, 148.9, 148.4, 136.1, 130.3, 124.3, 114.1, 70.8, 70.5, 70.4, 69.7, 68.6, 64.2, 46.8, 43.3, 36.6, 11.3; IR (KBr disc): $\nu_{\text{bar}}$ = 3358, 3212, 3054, 2915, 1690, 1640 cm$^{-1}$; HRMS (ESI): $m/z$ calcd for C$_{33}$H$_{56}$N$_8$O$_1$I: 899.3011 [M + H]$^+$; 899.2991 found. RP-HPLC: $R_t = 9.5$ min.

**Radiochemistry**

A solution of copper (II) chloride (134 µg, 1.0 µmol) and triethylamine (151 µg, 1.5 µmol) in acetonitrile (40 µL) was added to compound 9 (573 µg, 1.0 µmol). After 10 minutes the resulting solution was added to a mixture of compound 10 (199 µg, 1.0 µmol) in acetonitrile (20 µL) and Na$^{125}$I (9.8 MBq) in water (6 µL). The reaction was heated to 60°C for 90 minutes at which point the reaction was quenched with Na$_2$S$_2$O$_5$ (100 µL, 0.1M). The reaction mixture was purified using RP-HPLC, where the product was isolated in 36% radiochemical yield (3.6 MBq) in greater than 95% radiochemical purity.
Chapter 4: Pretargeting strategies for imaging melanoma using the tetrazine-\textit{trans}-cyclooctene reaction

4.1 Introduction

Having had limited success achieving high melanoma tumour uptake using the TAAG construct, an alternative approach was investigated which employed pre-targeting and bioorthogonal chemistry. Bioorthogonal reactions can be defined as “chemical reactions that neither interact with nor interfere with a biological system”.\textsuperscript{53} They must result in rapid formation of a covalent bond, while remaining chemically inert to other compounds present in the living system.\textsuperscript{54} There are only a few known reactions that can be considered bioorthogonal since there are many reactive functional groups present in living systems, and there are few reactions that would not be influenced by these species.

Another requirement is biocompatibility since the reactions are performed \textit{in vivo}. As a result typical click reactions such as the copper catalyzed Huisgen 1,3-dipolar cycloaddition between an azide and an alkyne are not an option due to the toxicity of copper.

Many bioorthogonal reactions have been investigated such as the Staudinger and strain promoted cycloadditions. One method that has garnered particular attention is the ligation between \textit{trans}-cyclooctene (TCO) and tetrazines. The tetrazine reacts with the TCO through a rapid inverse-electron-demand Diels-Alder reaction, with rate constants ranging from 400 to 2000 M\textsuperscript{-1}s\textsuperscript{-1}.\textsuperscript{55} This is sufficiently rapid to allow tetrazines to couple...
with cells tagged with TCO-derived vectors. The advantage here is that visualization at
the site of interest with minimal background uptake can be achieved. This approach
alleviates much of the non-target radiation dose received by the patient as the TCO
labelled probe has already cleared the non-target tissue when the radiolabel is
administered.

A recent paper showed the injection of a TCO labelled CC49 antibody to target
human colon cancer xenografts.\(^{56}\) Antibodies are an ideal system for the TCO tetrazine
ligation as typically antibodies circulate in the blood for greater than 24 hours, and result
in high tumour uptake.\(^{57}\) The TCO-antibody conjugate was injected into mice bearing a
colon cancer xenograft, and allowed to circulate for 24 hours, at which point an \(^{111}\)In
labelled tetrazine ligand was administered and the subjected mice imaged 3 hours post
injection. There was excellent tumour visualization and low background uptake in
comparison to the control, where no TCO-antibody was administered (Figure 22).\(^{56}\)
Another example of the pretargeting strategy using the tetrazine-TCO ligation was
performed in the Valliant group, where a TCO-anti-VEGFR2 antibody was used to
capture ultrasound contrast agents.\(^{58}\) Here a TCO labelled antibody was injected 24 hours
prior to the administration of the tetrazine to allow for tumour accumulation. Through the
use of tetrazine labelled microbubbles the tumour was visualized with a greater level of
contrast than the control which had no TCO-antibody present.
Figure 22: Small-animal SPECT/CT imaging of live mice bearing colon carcinoma xenografts: posterior projections of mice preinjected with a) CC49-TCO (100 µg) followed one day later by $^{111}$In-ligand (25 equiv to CC49; 3.4 equiv to TCO, 42 MBq), b) CC49 (100 µg) followed one day later by $^{111}$In-I (same amount as in (a), 20 MBq), and c) Rtx-TCO (100 mg) followed one day later by $^{111}$In-I (same amount as in (a), 50 MBq)\(^{56}\)

The promising results, fast reaction kinetics, stable product, and \textit{in vivo} selectivity make the TCO-tetrazine ligation an attractive system for the development of a melanin specific therapy agent. The basic concept would involve using a TCO-derived benzamide to tag melanin followed by administration of a radiolabelled tetrazine. With respect to the choice of targeting construct, $^{125}$I-BZA had approximately three times the tumour uptake at 24 hours than the TAAG derivatives. In the literature it has been shown that BZA demonstrates high non-specific binding at early time points, but clears most non-target
tissue by 24 hours. The development of a pre-targeted probe based on the BZA core rather than the TAAG constructs was therefore selected.

4.2 Results and Discussion
4.2.1 Synthesis of a TCO- Derived Benzamide

It has been shown in previous work that the procainamide core can be derivatized and still retain strong binding to melanin (Figure 23). One specific example MIP-1145, which was mentioned earlier, showed high uptake of 8 %ID/g in melanoma murine models and was recently advanced to phase I clinical trials. A more recent example is $^{131}$I-BA52, which has the same core as MIP-1145, was used in an imaging and therapy study on patients with metastatic melanoma with promising results. The promising results of procainamide derivatives functionalized at the aniline nitrogen suggested that a TCO could be introduced into the benzamide core without detrimental impact on binding.

Compound 13 (Scheme 6) was synthesized from the TCO-carbonate active ester using DMAP, procainamide hydrochloride and DIPEA. The reaction was stirred at room temperature for 7 days at which point the mixture was evaporated and purified using semi-preparative HPLC. The compound was characterized by $^1$H and $^{13}$C NMR, HRMS, and HPLC.
4.2.2 Ligation

To prove that the benzamide-TCO derivative would still couple with a tetrazine, a solution of (13) (1mg/mL) in saline was combined with 14 (Scheme 7), which is a known tetrazine. The test was done with excess tetrazine to ensure that the reaction would go to completion. The result of this study showed the appearance of a new peak in the HPLC and disappearance of the starting material peak within 5 minutes after incubation of the TCO and tetrazine compounds (Figure 25). To prove that the compound was in fact undergoing the coupling reaction and not decomposing, a high resolution mass spectrum of the solution was acquired. The resulting spectrum shows a peak at 596.3356, which is within 1.2 ppm of the theoretical model, confirming the new peak in the HPLC corresponds to the TCO-tetrazine complex, and not a decomposition product.
Figure 24: High resolution ESI$^+$ mass spectrum of the TCO-tetrazine ligation

Scheme 7: Tetrazine TCO ligation.
4.2.3 In vitro Assays

An in vitro assay was performed with compound 13 to ensure that once the compound has bound cells the TCO group is still available to undergo the reverse-demand Diels-Alder reaction with a radiolabelled tetrazine (15). The tetrazine precursor was synthesized by Silvia Albu and radiolabelled by Denis Beckford, two members of the Valliant research group. The assay was performed using three cell lines, a high melanin expresser (B16), a medium melanin expresser (MeWo), and a non-melanin expresser (A431).62–65 Each of the cell lines were incubated with and without TCO, and to all the cells was added 15. The cells were lysed and the percent activity bound in each of the cell lines was measured. There was minimal retention of activity in the cells without 13.
showing little non-specific binding (Figure 26). The B16 cell line showed greater than 25% bound when incubated with 13, approximately three times the amount in the MeWo cells (~8%). This is the expected trend as with more melanin there is more target for the compound to bind, consequently the B16F1 tumours should show the highest uptake. The non-melanin expresser showed an intermediate uptake around 15%, which was unexpected considering there is no target expressed in these cells. This binding is likely the result of non-specific interactions which could promote poor target-to-non-target ratios in vivo. Nevertheless, due to the promising trend seen with the high and moderate melanin expressers 13 and 15 were used in a biodistribution study.
4.2.4 Biodistribution Studies

There are two time parameters to consider for this study, how long after the TCO is administered should the tetrazine be administered and at what time points should the tissues be counted after the injection of the labeled component. To determine the optimal time point for sacrifice and counting, a biodistribution study was performed using 15 in healthy C57Bl/6 mice. Two time points were chosen for the biodistribution study, 6 and 24 hours post injection with three mice per time point. The biodistribution data showed that by 24 hours 15 had cleared most non-target tissues such as the eyes, large intestine, and gallbladder, which still had high uptake at 6 hours of 1.9, 38, and 25 respectively (Figure 27). Based on these results the ideal time point for the tetrazine circulation is 24 hours, which will be applied to the combined BZA-TCO and tetrazine biodistribution.
Once the optimal time for tetrazine circulation was determined, a biodistribution study was conducted using two groups of 3 mice. Both groups used B16F1 bearing C57Bl/6 mice, the control group were injected with 15 while the test group received 15 after a 4 hour incubation of 400 µg of 13. The results of the study showed that there was a difference between the group with and without TCO, specifically the amount in the tumour and the eyes which both express melanin (Figure 28). The uptake in the eyes was 6 and 0.8 ID/g in the TCO containing group and the control group respectively. The tumour uptake was 0.7 and 0.1 %ID/g in the TCO containing group and the control group respectively. These results are very promising as there is a difference between the group with TCO and without, however there is a very large error associated with the TCO group.

Figure 27: Biodistribution results for 15 in healthy C57Bl/6 mice

![Biodistribution results](image)
therefore the experiment needs to be repeated with a larger sample size. That being said, the uptake in the tumour is still less than ideal for therapy and potentially derivatizing the procainamide backbone with an iodine as well as a methoxy group (as seen in MIP-1145 and BA52) could help increase tumour uptake.

Figure 28: Biodistribution data for 13 and 15 (grey) and the control 15 (black)

4.3 Conclusions

A TCO-BZA derivative was synthesized, characterized, and evaluated in vivo for its ability to target melanin. The studies performed here used a simple tetrazine for the purpose of determining the tumour uptake of the BZA-TCO complex, however this specific tetrazine would not likely be used clinically due to the slow clearance from non-
target tissue. The preliminary studies of the TCO-BZA construct with $^{125}\text{I}-\text{Tz}$ have shown promising results, however for this approach to be clinically relevant to melanoma an increase in tumour uptake of the TCO-Tz will have to be accomplished, potentially by further functionalizing the vector to enhance uptake.

4.4 Experimental

$^1\text{H}$ and $^{13}\text{C}$ NMR spectra were recorded on a Bruker AV 600 spectrometer. High Resolution Mass Spectra (HRMS) were recorded on a Waters Micromass QTof Global mass spectrometer using electrospray ionization (ESI). SiliaFlash P60 Silica gel from SiliCycle was used for silica gel chromatography. All reagents were purchased from Sigma-Aldrich and Click chemistry tools and used without further purification. High pressure liquid chromatography (HPLC) was performed using a Varian ProStar Model 230 instrument, fitted with a Varian Pro Star model 330 PDA detector monitoring at 254 nm with a model 230 delivery system. Purification was performed using a Waters X-bridge C18 (5 µm x 10 x 100 mm) column with a binary solvent gradient (4 mL/min) consisting of 90% eluent A for 3 minutes to 0% eluent A over the next 13 min. Eluent A: water containing 0.1% TFA; Eluent B: ACN containing 0.1% TFA (method A).
Synthesis of (E)-cyclooct-4-en-1-yl (4-((2-diethylamino)ethyl)carbamoyl)phenyl carbamate (13)

To a vial of TCO-carbonate active ester (0.015 g, 0.06 mmol) in 1 mL of DMF was added DMAP (0.014 g, 0.12 mmol), procainamide hydrochloride (0.047 g, 0.17 mmol) and DIPEA (0.03 mL, 0.17 mmol). The reaction was stirred at room temperature for 7 days at which point the mixture was evaporated and dissolved in 5:1 water:ACN and purified using semi-preparative HPLC. The desired peak ($R_t = 14.0$ min) was collected, the ACN removed through rotary evaporation at which point the remaining water was frozen and put on the lyophilizer overnight to give compound 13 as a white powder (30 mg, 60%).

$^1$H NMR (600 MHz, D$_2$O) δ 7.79 (d, 2H, $J = 8.9$ Hz), 7.49 (d, 2H, $J = 8.9$ Hz), 5.73 (m, 1H), 5.61 (m, 1H), 4.44 (m, 1H), 3.43 (t, 2H, $J = 5.9$ Hz), 3.33 (m, 4H), 2.38 (m, 3H), 2.00 (m, 4H), 1.70 (m, 3H), 1.33 (t, 6H, $J = 7.3$ Hz); $^{13}$C NMR (150 MHz, MeOD) δ 171.0, 155.2, 144.6, 136.1, 133.9, 129.5, 128.0, 118.8, 82.3, 53.4, 42.1, 39.7, 36.5, 35.1, 33.1, 9.2;

HRMS (ESI): $m/z$ calcd for C$_{22}$H$_{33}$N$_3$O$_3$H: 388.2600 [M + H]$^+$; found. 388.2594; HPLC $R_t$ (method A): 14.0 min.
Chapter 5: Dendrimer Based Probes and Future Work

5.1 Introduction to Dendrimers

A potential strategy around which to develop a platform for melanin targeted imaging and radiotherapy is to exploit dendrimers. A dendrimer is a precisely defined polymer that can be functionalized at the core, periphery, and/or branching points. Dendrimers may be advantageous when compared to small molecules for melanoma targeted agents due to the potential of using multiple targeting vectors around the periphery of the polymer. At physiological pH melanin possesses multiple negative charges; the tertiary amine targeting vector relies on electrostatic interaction with the negatively charged groups on melanin. A dendrimer functionalized with multiple targeting vectors therefore has the potential for multiple electrostatic interactions and should increase the retention of the agent.

As an example, a dendrimer core was used to image angiogenesis using $^{76}$Br labelled CRGDC. This cyclic peptide is known to target $\alpha_v\beta_3$ integrin, which is over expressed in angiogenesis. The CRGDC peptide was functionalized with a lysine residue, which was subsequently linked to a dendrimer backbone. The dendrimer consisted of eight tyrosine residues, which can be directly radiolabelled with iodine or bromine. A PET imaging study was performed on the $^{76}$Br labelled dendrimer, and the results showed an increased uptake in the targeted approach compared to the control. These results were promising as they show the targeted dendrimer model can be successful in vivo compared to the non-targeted counterpart.
As a proof of concept study, $^{125}$I-7g (Figure 29), the aminobenzyl piperidine (ABP) targeting vector was conjugated to a first generation PMPA dendrimer (Scheme 4). This dendrimer was selected because previous work has been done that showed increased reactivity towards primary amines when the periphery was functionalized with $p$-nitrophenyl carbonate groups. The previous work involved functionalization of multiple generations of dendrimer with DFO to target infection. The work showed rapid reaction times, as well as successful deprotection of the dendrimer core. Based on these results, a melanin targeted generation one dendrimer was synthesized with ABP targeting vectors as it was the most successful TAAG derivative. Prior to preparing higher generation dendrimers, the approach focused on developing the methods needed to prepare the radiolabelled dendrimers containing two benzamide constructs to determine if there was any benefit to multi-valent binding.

![Figure 29: Structure of the lead TAAG derivative $^{125}$I-7g](image)

### 5.2 Results and Discussion

PMPA dendrimers were synthesized by Danielle Ellis in the Adronov research group according to literature procedures with a $p$-toluenesulfonyl ester carboxylic acid protecting group at the core. The periphery of the dendrimer was functionalized with $p$-nitrophenyl chloroformate to increase the reactivity of these groups toward primary
amines. The $p$-nitrophenyl carbonate functionalized generation 1 dendrimer was combined with aminobenzyl piperidine (ABP) to produce a functionalized dendrimer through a carbamate linkage (Scheme 8). The product was isolated in 28 % yield where the presence of the targeting vectors was apparent by comparing the integration of the protons on the targeting vector to the protons on the core of the dendrimer.

The dendrimer was deprotected at the core using DBU to give 19. It was subsequently conjugated to a TAAG derivative through an amide linkage (Scheme 9) to generate 21 in 27 % yield. Based on the integrations of the number of protons on the aryl ring compared to that for the triazole, it was apparent that the dendrimer had been functionalized with two targeting vectors. From the integrations, a 10:1 ratio exists between the benzyl protons and the triazole proton, which is consistent with the dual-functionalized structure. Furthermore, the high resolution mass spectrum also shows the correct mass of 1036.5441 which is consistent with the desired structure (Figure 30).
Scheme 8: Synthesis of TSeG1 dendrimers

Scheme 9: Synthesis of the dendrimer radiolabelling precursor
Once the precursor 21 had been fully characterized, an iododestannylation reaction was performed using $^{125}$I, and iodogen as the oxidizing agent. The solution was shaken at room temperature for 10 minutes, quenched with 100 µL of 0.1M Na$_2$S$_2$O$_5$, and purified using RP-HPLC (Scheme 10). The desired peak was isolated, the mobile phase evaporated, and the $^{125}$I radiolabelled compound 22 reconstituted in PBS for biological purposes (Figure 31). The radiolabelled dendrimer was co-injected with the non-radioactive standard to prove its identity (Figure 32).

Scheme 10: Radiolabelling Reaction
Figure 31: Purified gamma HPLC trace of $^{125}$I-22

Figure 32: HPLC trace of coinjected 22 and $^{125}$I-22 UV chromatogram 254 nm (top) gamma chromatogram (bottom)
The log P of $^{125}$I-22 was determined to be 1.2 ± 0.3 using the shake flask method. The stability of $^{125}$I-22 in PBS was tested, and found to be stable at least 11 days (Figure 33).

![Gamma HPLC chromatogram of $^{125}$I-22 11 days after formulation](image)

Figure 33: Gamma HPLC chromatogram of $^{125}$I-22 11 days after formulation

A biodistribution study was performed on $^{125}$I-22 in C57Bl/6 mice bearing B16F10 mice at two time points, 4 and 24 hours. Unfortunately there was little uptake in the tumour (0.07 % ID/g) compared to $^{125}$I-7g (2.2 %ID/g). The biodistribution data (Figure 34) shows high uptake in the gallbladder (70 %ID/g) and large intestine (88 %ID/g) at 4 hours. These results are similar to what was seen with $^{125}$I-11, another high molecular weight compound. The difference in log P of the two compounds is around 1 log unit, therefore the difference in polarity of the two compounds is a factor of ten. Neither $^{125}$I-11 or $^{125}$I-22 showed ideal pharmacokinetic properties, and the polarity of the two compounds was substantially different, it can be concluded that the large molecular weight of these compounds may be impacting clearance in an less than ideal fashion.
Along with clearance, it is possible that steric hindrance about the tertiary amine due to the carbamate linkage and the phenyl rings is affecting the ability of the probe to bind melanin. If the targeting vector portion of the molecule is not able to orient properly it may not be able to successfully bind to the target. To determine if the low tumour uptake was due to steric hindrance, the next compound that was synthesized contained DEED targeting vectors (Scheme 8). As the phenyl rings will no longer be present the compound should be able to bind more easily to the melanin. The TSe protected G1 DEED derivative was synthesized, however attempts to deprotect and purify were not successful as the compound is very polar and is not UV active, which made purification challenging and ultimately unsuccessful.
5.4 Conclusion

A melanin targeted generation one dendrimer was synthesized and evaluated *in vivo* for its ability to target melanoma tumours. The results from the biodistribution study showed that the compound would not be clinically useful. Due to, time constraints the DEED and BZA derivatives were not tested *in vivo* but offer a potential solution to the rigidity issue seen with the ABP derivative. The dendrimer platform offers versatility for imaging and therapy agents as it is not restricted to melanoma, as well there are additional strategies that can be followed to functionalize the periphery and the core as a means of addressing the limitations observed.

5.5 Future Work: Integration of the dendrimer and TCO platforms

Although the first dendrimer was not successful, one potential option is to functionalize the core of the dendrimer with trans-cyclooctene (TCO), which allows for a bioorthogonal click reaction with a tetrazine. This would be useful for surgical resection of primary melanomas, as many times it is difficult for the surgeon to identify the boarders of the cancerous tissue. By creating a targeted compound with a TCO group, a patient can be administered the agent before surgery, and the surgeon can apply a tetrazine dye which would fluoresce if it reacted with a TCO group, indicating the presence of cancer cells (Figure 35).14
The dendrimer platform allows for easy functionalization of the periphery with any primary amine, as well as the ability to functionalize at the core. Due to the simplicity of the coupling reactions, this can be extended to other targeting vectors and allows for imaging or therapy of many different processes in vivo.

5.6 Future work: BZA-TCO

With respect to TCO derivatives, functionalizing the TCO-BZA backbone could help increase the binding and retention to a melanin positive tumour. In the literature there have been multiple examples where functionalizing the phenyl ring present in BZA has been shown to increase the uptake in the tumour relative to native BZA. Derivatives of the TCO-BZA core could be synthesized to try to increase the tumour uptake; one potential derivative is shown in Figure 36. This is based on the recent observation that the addition of methoxy group to benzamides can enhance melanin binding and retention.\textsuperscript{27,45} Another way to derivatize the core would be to add a spacer between the TCO group and the BZA, which would allow fine-tuning of the lipophilicity as well as make the TCO group more accessible to the injected tetrazine.
5.7 Experimental

*Synthesis of 2-tosylethyl 3-(((1-benzylpiperidin-4-yl)carbamoyl)oxy)-2-(((1-benzylpiperidin-4-yl)carbamoyl)oxy)methyl)-2-methylpropanoate (16)*

A 5 mL microwave vial equipped with a magnetic stir bar was charged with TSe-G1-(p-nitrophenyl carbonate) (0.331 g, 0.512 mmol) in DCM (2.0 mL). To the mixture was added 4-aminobenzyl piperidine (0.420 mL, 2.05 mmol) and DIPEA (0.356 mL, 2.05 mmol), the reaction mixture was stirred for 24 hours. The reaction mixture was concentrated and purified by silica gel chromatography (100 % EtOAc 3 CV, 10 :1 DCM/MeOH), the desired fractions were concentrated to give compound 16 (0.109 g, 28%) as a yellow powder. $^1$H NMR (600 MHz, CDCl$_3$) 7.79 (d, 2H, $J = 8.5$ Hz), 7.73 (s, 2H), 7.70 (d, 4H, $J = 8.4$ Hz), 7.44 (d, 4H, $J = 8.0$ Hz), 7.34 (d, 2H, $J = 8.0$ Hz), 7.01 (s, 2H), 4.47 (t, 2H, $J = 5.5$ Hz), 4.29 (m, 4H), 3.46 (m, 6H), 2.66 (t, 4H, $J = 5.9$ Hz), 2.58 (q, 8 H, $J = 8.0$ Hz), 2.42 (s, 3H), 1.23 (s, 3H), 1.03 (t, 12 H, $J = 7.1$ Hz); $^{13}$C NMR (150 MHz, CDCl$_3$) δ 172.9, 155.1, 145.3, 136.1, 130.2, 129.2, 128.3, 128.1, 127.2, 65.8, 62.9, 58.0, 55.1, 52.0, 48.2, 46.9, 32.2, 21.7, 17.2. HRMS (ESI): $m/z$ calcd for C$_{40}$H$_{52}$N$_{4}$O$_{8}$SH: 749.3584 [M + H]$^+$; found 749.3561.
**Synthesis of 2-tosylethyl 3-(((2-(diethylamino)ethyl)carbamoyl)oxy)-2-(((2-(diethylamino)ethyl)carbamoyl)oxy)methyl)-2-methylpropanoate (17)**

A 5 mL microwave vial equipped with a magnetic stir bar was charged with TSe-G1-(p-nitrophenyl carbonate)\(_2\) (0.188 g, 0.291 mmol) in DCM (1.0 mL). To the mixture was added N,N-diethylethylene diamine (0.163 mL, 1.16 mmol) and DIPEA (0.200 mL, 1.16 mmol), the reaction mixture was stirred for 24 hours. The reaction mixture was diluted with 15 mL of DCM and washed with 0.01M HCl solution, followed by washing with saturated sodium bicarbonate solution (2 x 15 mL). The bicarbonate layers were back extracted with 20 mL of ethyl acetate which was combined with the DCM layers, dried over sodium sulfate and concentrated. The organic layer was purified through silica gel chromatography (100:10:1 v/v/v CHCl\(_3\):MeOH:NH\(_4\)OH increasing to 80:15:1.5 v/v/v CHCl\(_3\):MeOH:NH\(_4\)OH). The desired fractions were concentrated to give compound 17 (0.077 g, 44%) as a yellow oil. \(^1\)H NMR (600 MHz, MeOD) \(\delta\) 7.83 (d, 2H, \(J = 8.0\) Hz), 7.47 (d, 2H, \(J = 8.0\) Hz) 4.41 (t, 2H, \(J = 5.5\) Hz), 4.02 (m, 4H), 3.58 (t, 2H, \(J = 5.5\) Hz), 3.35 (s, 3H), 3.20 (t, 4H, \(J = 7.0\) Hz), 2.62 (m, 12H), 2.46 (s, 3H), 1.07 (t, 12H, \(J = 7.0\) Hz); \(^1\)C NMR (150 MHz, MeOD) \(\delta\) 158.3, 146.7, 131.3, 129.3, 66.7, 59.4, 55.85, 53.0, 48.2, 39.1, 21.6, 17.5, 11.4. HRMS (ESI): \(m/z\) calcd for C\(_{28}\)H\(_{48}\)N\(_4\)O\(_8\)S\(_2\): 601.3271 [M + H]\(^+\); found 601.3281.
Synthesis of 2-tosylethyl 3-(((4-((2-(diethylamino)ethyl)carbamoyl)phenyl)carbamoyl)oxy)-2-(((4-((2-(diethylamino)ethyl)carbamoyl)phenyl)carbamoyl)oxy)methyl)-2-methylpropanoate (18)

A 10 mL microwave vial equipped with a magnetic stir bar was charged with TSe-G1-(p-nitrophenyl carbonate)₂ (0.389 g, 0.602 mmol) in DCM (9.0 mL). To the mixture was added procanamide hydrochloride (0.654 g, 2.41 mmol), DIPEA (0.418 mL, 2.41 mmol), and DMAP (0.073 g, 0.602 mmol) the reaction mixture was stirred for 24 hours. The reaction mixture was diluted with 15 mL of DCM and washed with 0.01M HCl solution, followed by washing with saturated sodium bicarbonate solution (2 x 15 mL). The organic layer was subsequently dried over sodium sulfate and concentrated. The organic layer was purified through silica gel chromatography (100:10:1 v/v/v CHCl₃:MeOH:NH₄OH and the desired fractions were concentrated to give compound 18 (0.131 g, 26%) as a yellow oil. ¹H NMR (600 MHz, CDCl₃) 7.77 (d, 2H, J = 8.0 Hz), 7.73 (s, 2H), 7.70 (d, 4H, J = 8.5 Hz), 7.44 (d, 4H, J = 8.4 Hz), 7.34 (d, 2H, J = 8.0 Hz), 7.01 (s, 2H), 4.47 (t, 2H, J = 5.5 Hz), 4.29 (m, 4H), 3.46 (m, 6H), 2.66 (t, 4H, J = 5.9 Hz), 2.58 (q, 8H, J = 8.0 Hz), 2.42 (s, 3H), 1.23 (s, 3H), 1.03 (t, 12 H, J = 7.1 Hz); ¹³C NMR (150 MHz, CDCl₃) δ 172.8, 166.8, 152.8, 145.5, 140.9, 135.9, 130.3, 129.3, 128.0, 127.9, 118.0, 66.4, 58.0, 55.2, 51.4, 46.9, 37.2, 21.7, 17.3, 11.8. HRMS (ESI): m/z calcd for C₄₂H₅₈N₆O₁₀SH: 420.2040 [M + 2H]²⁺; found 420.2037.
**Synthesis of 3-(((1-benzylpiperidin-4-yl)carbamoyl)oxy)-2-(((1-benzylpiperidin-4-yl)carbamoyl)oxy)methyl)-2-methylpropanoic acid (19)**

To a solution of 16 (0.100 g, 0.133 mmol) in DCM (1 mL) was added DBU (0.100 mL, 0.668 mmol) and the reaction stirred at room temperature for 5 hours. The reaction mixture was concentrated, dissolved in 1 mL of 70/30 water/acetonitrile and loaded onto an activated C18 cartridge. The cartridge was washed with 5 mL of 70/30 water/ethanol, at which point the desired product was eluted with 10 mL of 50/50 water/ethanol. The desired fractions were concentrated to give compound 19 (0.054 g, 71%).

**Synthesis of 2-methyl-2-((4-((2-(4-(tributylstannyl)-1H-1,2,3-triazol-1-yl)acetamido)butyl)carbamoyl)propane-1,3-diyl bis((1-benzylpiperidin-4-yl)carbamate) (21)**

To a 5 mL microwave vial containing 19 (0.020 g, 0.035 mmol) dissolved in 2 mL of DMF was added 20 (0.017 g, 0.035 mmol), HATU (0.020 g, 0.053 mmol), and DIPEA (0.025 mL, 0.142 mmol). The reaction mixture was stirred at room temperature for 22 hours at which point it was concentrated and purified on a 10 g SNAP cartridge (0-20% DCM MeOH 40 CV. Compound 21 was isolated as a yellow oil (27%, 10 mg). $^1$H NMR (600 MHz, MeOD) 7.93 (s, 1 H), 7.32 (m, 8H), 7.28 (m, 2H), 5.16 (s, 2H), 4.15 (s, 4H), 3.59 (s, 4H), 3.38 (m, 2H), 3.22 (m, 4H), 2.90 (m, 4H), 2.21 (m, 2H), 1.85 (m, 4H), 1.55 (m, 14H), 1.34 (m, 6H), 1.20 (s, 3H), 1.15 (m, 6H), 0.90 (t, 9H, $J=7$ Hz); $^{13}$C NMR (150 MHz, CDCl$_3$) δ 173.1, 166.0, 155.4, 145.5, 131.9, 129.3, 128.3, 127.3, 67.1, 62.8, 52.6,
52.1, 48.2, 46.6, 39.4, 38.7, 32.1, 29.0, 27.8, 27.4, 27.2, 27.0, 26.3, 26.0, 18.0, 13.7, 10.0.

HRMS (ESI): $m/z$ calcd for C$_{51}$H$_{81}$N$_9$O$_6$Sn: 1036.5422 [M + H]$^+$; 1036.5441 found.

Synthesis of $2\text{-}((4\text{-}(2\text{-}(4\text{-}iodo\text{-}1H\text{-}1,2,3\text{-}triazol\text{-}1\text{-}yl)acetamido)butyl)carbamoyl)\text{-}2\text{-}methylpropane\text{-}1,3\text{-}diyl bis((1\text{-}benzylpiperidin\text{-}4\text{-}yl)carbamate)$ (22)

To a solution of 21 (100 µg) in chloroform (100 uL) was added iodogen (25 µg) in chloroform (25 µL) and acetic acid (5 µL). The reaction was placed on the shaker for 10 minutes at which point it was quenched with Na$_2$S$_2$O$_5$ (0.1 M, 100 µL). The aqueous layer of the reaction mixture was then purified by RP-HPLC, the desired peak collected, concentrated, and formulated in PBS for biological studies. Compound 22 was isolated in greater than 95% RCP, in 19% RCY. HPLC Rt (Method A): 8.30 minutes.

References


6. Jeffrey A. Sosman, M.D., Kevin B. Kim, M.D., Lynn Schuchter, M.D., Rene Gonzalez MD, Anna C. Pavlick, D.O., Jeffrey S. Weber, M.D., Ph.D., Grant A. McArthur, M.B.,


