MOLECULAR IMAGING AGENTS FOR RHEUMATOID ARTHRITIS AND CANCER
SYNTHESIS OF NOVEL PET AND FLUORESCENT PROBES FOR THE MOLECULAR IMAGING OF RHEUMATOID ARTHRITIS AND CANCER

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Abstract

This thesis describes novel molecular imaging strategies that can ultimately be used for imaging rheumatoid arthritis (RA) and cancer. In the first approach, a convenient method for the preparation of a “turn on” near-infrared (NIR) optical reactive oxygen species (ROS) probe and its $^{18}$F analogue is described. The probe and its radiolabeling precursor are prepared in high yield (82 and 98% respectively) via a Suzuki coupling in two steps. Chemical induction studies revealed the probe selectively responds to hydroxyl and superoxide radicals. The PET probe was accessed via a late-stage fluorination approach from a nitopyridine precursor in a 62% decay-corrected radiochemical yield. As a proof of concept, the PET probe was assessed in vivo in C57BL/6 mice using a doxorubicin induced model of cardiac ROS which revealed a statistically significant retention in the heart as compared to untreated mice ($p < 0.05$). The probe additionally demonstrated high-blood retention in vivo, which is likely due to albumin binding. Less hydrophobic derivatives of this probe were synthesized in a similar approach with the ultimate goal of improving in vivo clearance.

Building on this work, the synthesis of a meso fluorinated cyanine dye derived from copper-catalyzed halogen exchange of IR780 iodide is discussed. Optimized reaction conditions led to a 68% yield in the presence of a phosphaadamantane ligand and a copper (II) catalyst. The fluorinated probe exhibited enhanced fluorescence in comparison to IR780 in solution. The corresponding ROS probes (hydrocyanines) were assessed in a PC-3 cell assay where the fluorinated probe
exhibited 2-3 fold fluorescence enhancement. Radiolabeling to produce the $^{18}$F analogue was attempted, but this was found to be irreproducible. This fluorinated dye along with IR780 and other synthesized fluorescent probes were encapsulated in F-127 micelles which resulted in the preparation of monodispersed micelles (PDI ≤ 0.2). Improved solubility and fluorescence were observed as compared with unencapsulated dyes. An MTT assay performed on the encapsulated ROS probe revealed it was considerably less cytotoxic than the parent probe.

Alternatively, a targeted approach via the development of molecular probes based on two classes of high affinity TACE enzyme inhibitors is described. Three compounds were synthesized and assessed in a fluorogenic assay in which one compound demonstrated binding in the micromolar range (IC$_{50}$ = 1770 nM) while the remaining compounds demonstrated low nanomolar affinity (IC$_{50}$ = 5-10 nM). Intermediates that could serve as precursors in $^{18}$F labeling via a prosthetic group labeling strategy were prepared.
Acknowledgements

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<tr>
<td>[18F]FAZA</td>
<td>18F-fluoroazomycin arabinoside</td>
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<tr>
<td>[18F]DOPA</td>
<td>18F fluorodihydroxyphenylalanine</td>
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<td>[18F]FDG</td>
<td>2-deoxy-2-[18F]-fluoro-D-glucose</td>
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<td>[18F]FLT</td>
<td>18F-fluorothymidine</td>
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<tr>
<td>β-APP</td>
<td>beta amyloid plaque</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>BSA</td>
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<td>DLS</td>
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<td>disease-modifying anti-rheumatic drugs</td>
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<td>dimethylformamide</td>
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<td>EE</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<td>ELISA</td>
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<td>eNOS</td>
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<td>enhanced permeability and retention</td>
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<td>FDA</td>
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<td>GSH</td>
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<td>HALEX</td>
<td>halogen exchange</td>
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<tr>
<td>Acronym</td>
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<tr>
<td>HEPES</td>
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<td>HOBT</td>
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<td>HPLC</td>
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<tr>
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<td>trifluoroethylformate</td>
</tr>
<tr>
<td>THP</td>
<td>tetrahydropyranyl</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>US</td>
<td>ultrasound</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>Val</td>
<td>valine</td>
</tr>
<tr>
<td>Γ</td>
<td>gamma</td>
</tr>
<tr>
<td>ΔΨm</td>
<td>mitochondrial membrane potential</td>
</tr>
<tr>
<td>β⁺</td>
<td>positron</td>
</tr>
<tr>
<td>N</td>
<td>neutrino</td>
</tr>
</tbody>
</table>
Declaration of Academic Achievement

As part of the NSERC CREATE molecular imaging probes program, I worked alongside scientists of various disciplines in an effort to develop molecular imaging probes for the detection and characterization of rheumatoid arthritis (RA). My PhD work describes new platforms that can be used to create tools for imaging this disease. Firstly, I developed the first known example of a radiolabeled (\(^{18}\text{F}\)) hydrocyanine dye, which are a unique class of probes that are capable of fluorescing in the near-infrared region in the presence of elevated ROS levels. In this way, the probe can be utilized as a dual modality probe for either optical imaging or PET imaging. This probe exhibits superior stability and can be accessed via a late-stage fluorination approach. While performing the biological studies, it was found that the probe exhibited exceptional blood-binding properties which preliminary studies suggest is due to albumin binding.

In terms of contribution, I initiated and developed the project. I synthesized the probes and radiolabeling precursors in addition to performing optical studies, stability studies and all experiments related to radiolabeling. I developed synthetic and radiosynthetic protocols and optimized all conditions for these probes. Characterization was performed by myself and Dr. Silvia Albu. Alyssa Vito and I designed and performed albumin binding studies. Shannon Czorny performed \textit{in vivo} experiments in addition to the optical cell imaging studies and the toxicity study. Nancy Janzen performed \textit{in vivo} studies and a PC-3 based fluorescence assay. Despite not directly handling the mice and cells, I was involved in
experimental design and data acquisition including statistical analyses and quantification of optical fluorescence. Laura Banevicious performed preliminary excitation and emission spectra. Dr. Max Nanao and Dr. Jon Zubieta generated the X-ray crystallography data. Dr. John Valliant and Dr. Alfredo Capretta are the principle investigators and led the research efforts. The work described in Chapter 2 has been prepared as a manuscript for submission to Chemistry- A European Journal. As an extension of this work, I also synthesized less hydrophobic derivatives and performed optical studies and characterization. A precursor for one of the probes was synthesized and the compound was successfully $^{18}$F-labeled.

Secondly, F-127 micelle-encapsulated fluorophores were prepared with synthesized fluorophores and commercially available IR780. Similarly, these agents are capable of accumulating in areas of hypervascularity which is applicable for the assessment of RA but can also be used in theranostic approaches. One of the fluorophores that were used in the encapsulation procedure was a meso fluorinated analogue of IR780, which was accessed via a one step halogen exchange reaction from IR780. This compound showed enhanced fluorescence in solution and the reduced derivative accessed by reduction of the iminium bond demonstrated greater fluorescence response in cells by comparison to the chlorinated dye. Currently, this is the first example of a meso fluorinated cyanine dye. For this compound, I performed all synthetic work, optimization, radiolabeling, characterization and optical studies. Nancy Janzen performed the PC-3 cell assay with the respective reduced probes. For the micelle project, I performed the
encapsulation and purification of all fluorophores. I generated all calibration curves for the loading calculations and performed all optical studies described. Stuart McNelles performed dynamic light scattering (DLS) measurements and Dr. Carmen Andrei generated TEM images. Toxicity studies (MTT assay) were performed by Shannon Czorny.

Finally, the work also describes a targeted approach to imaging RA, which involves the synthesis of inhibitors for the metalloprotease enzyme TACE. Preliminary work described in this thesis includes the synthesis of hydroxamic and reverse hydroxamate inhibitors, *in vitro* assessment of the synthesized compounds and the synthesis of radiolabeling precursors. The scaffolds were synthesized using some procedures that varied from the reported literature protocols due to low yields and difficulty in accessing the compounds of interest. I performed the synthesis of all hydroxamic acid compounds and Dr. Ramesh Patel was involved in the scale-up synthesis of some intermediates. Blake Helka (in collaboration with Dr. John Brennan) was involved in preliminary assay optimization and Nancy Janzen performed the *in vitro* assay in addition to generating IC50 curves.

**Peer-reviewed Publications**

Chapter 1: Introduction

1.1 Molecular Imaging

“Molecular imaging” is defined as the noninvasive \textit{in vivo} visualization of biological processes at the cellular and molecular levels.\textsuperscript{1} This typically involves the construction of a 2 or 3 dimensional image capable of being quantified over time.\textsuperscript{2} Diagnostic medicine relies on molecular imaging techniques to provide information on the location and extent of a given disease. Typically, highly specialized instruments used solely or in combination with “molecular probes” are used to assess underlying pathology or mechanisms of a disease. Consequently, molecular imaging has shown utility and impact in clinical research and drug discovery in addition to being used for diagnostics in monitoring therapeutic response.\textsuperscript{3}

Generally, molecular imaging probes can be classified as being used for a “direct” imaging approach which involves the development of probes that bind or cross cell membranes and become trapped intracellularly.\textsuperscript{4,5} Images constructed with the aid of such probes rely on signal intensity proportional to the local concentration of the probe.\textsuperscript{8} Successful probe design relies on specific criteria such as accessibility to the target, pharmacokinetics/dynamics, target affinity/specificity, probe stability, and low non-specific binding. These probes span from low (i.e. small molecule ligands) to high molecular weight entities (i.e. antibodies, recombinant proteins).
A number of molecular imaging modalities are routinely used in a clinical setting. These include magnetic resonance imaging (MRI), ultrasound (US), optical imaging (OI) and nuclear imaging. Nuclear imaging, which is the focus of the research described in this thesis, is comprised of two modalities; single photon emission computed tomography (SPECT) or positron emission tomography (PET).

1.1.1 SPECT

SPECT is a nuclear tomographic imaging technique that employs gamma-emitting radiotracers. These radiotracers are injected intravenously into the patient and emitted gamma rays are detected by a rotating gamma camera that ultimately generates a 3D image. The gamma rays pass through a lead collimator which omits photons that are not within linear view of the detector, and are ultimately converted into an electrical signal by a photodetector. Because of the need to use collimators, the sensitivity of the technique is compromised compared to PET. The most commonly used SPECT isotopes are $^{99m}$Tc, $^{131}$I, $^{111}$In, and $^{67}$Ga. As SPECT isotopes commonly used in clinical nuclear medicine typically possess longer half-lives than PET isotopes, SPECT images can be acquired hours after administration of the agent.

1.1.2 PET

PET is a nuclear imaging technique that relies on neutron deficient nuclides that emit positrons ($\beta^+$ decay). In $\beta^+$ decay, neutron deficient nuclei attempt to achieve stability through the simultaneous emission of a positron ($\beta^+$) and a neutrino ($\nu$); a
process that converts a proton into a neutron. The emitted positrons interact with electrons resulting in an annihilation event which results in the generation of two 511 keV γ-rays traveling at opposite directions (approximately 180° apart, Figure 1.1) that are detected by the PET camera.\(^8\) Signals from two photons originating from a single positron annihilation event are counted simultaneously by the detector (i.e. “a coincident event”) thereby eliminating the need for collimation.\(^2\) After image reconstruction, quantitative information such as the 3D tracer concentration localized within a specific organ can be obtained. Traditionally, PET isotopes are short-lived in comparison to SPECT isotopes. The most commonly used PET isotopes are \(^{11}\text{C}\) and \(^{18}\text{F}\), exemplified by the most commonly employed radiotracer \([^{18}\text{F}]\text{FDG}\).\(^9\) \([^{18}\text{F}]\text{FDG}\) (a fluorinated glucose analogue) targets areas of high glucose metabolism, making it effective at detecting the high metabolic rate experienced in tumours, inflammation and infection. Other clinically relevant tracers include intracellular detection of hypoxia via nitroimidazoles \([^{18}\text{F}]\text{MISO}\) and \([^{18}\text{F}]\text{FAZA}\), cellular proliferation via \([^{18}\text{F}]\text{FLT}\) in addition to various others currently in development.\(^10\)

While both PET and SPECT provide greater depth penetration and the capability of performing whole body imaging, PET is commonly regarded as the superior imaging modality in terms of sensitivity, resolution and quantitation when compared to SPECT. One key advantage is that PET exhibits an appreciatively higher sensitivity (by two to three orders of magnitude) due to a higher amount of detected emission events brought about by the absence of a collimator.\(^6\) Enhanced
sensitivity permits improved image quality, temporal resolution and the ability to acquire images in a shorter period of time.\textsuperscript{6}

\begin{center}
\textbf{Figure 1.1} Schematic representation of positron-electron annihilation and coincident events identified by gamma detectors.\textsuperscript{11}
\end{center}

\subsection*{1.1.3 Optical Imaging}

Optical imaging (OI) utilizes nonionizing photons ranging from visible to near infrared (NIR) wavelengths (650-900 nm).\textsuperscript{12} Several contrast mechanisms exist for OI techniques such as light absorption/scattering, bioluminescence and fluorescence.\textsuperscript{13} Briefly, bioluminescence imaging involves cells that are transfected to express the enzyme luciferase, where the emitted light is detected by a charge-coupled device (CCD) camera.\textsuperscript{14} The luciferase enzyme catalyzes conversion of the substrate D-luciferin to oxyluciferin, causing emission of green light at 562 nm.\textsuperscript{15} This approach does not require an external excitation source which ultimately leads to a superior signal-to-noise ratio.\textsuperscript{12} However, this imaging technique is limited to preclinical models as a result of the need to transfec cells.
Unlike bioluminescence, optical fluorescence imaging generally requires administration of a fluorescent agent. The applied external light source immediately causes photons (at longer wavelengths to the excitation wavelength) to be emitted by the localized fluorophore and subsequently detected by a CCD camera (Figure 1.2).\(^3\)

![Figure 1.2 A schematic representation showing the general principles of fluorescence OI (reproduced with permission according to CC-BY 4.0 licence).\(^3\)](image)

Fluorescence imaging at greater depths (several centimeters) requires administration of a fluorescent agent that emits in the NIR region of the spectrum (650-900nm).\(^{16}\) Since tissues do not autofluoresce at these wavelengths, signal-to-noise is significantly improved through the use of such agents.\(^{17}\) One major advantage of fluorescence imaging is that it is a highly sensitive technique, capable of detecting sub-picomolar concentrations.\(^{16}\) Despite this, optical techniques usually require higher concentrations of injectable probe by comparison with PET/SPECT which require nano- to picomolar concentrations of the agent.\(^3\)
While a number of advantages such as ease of use and low costs exist for OI, optical techniques suffer from major drawbacks in comparison to other molecular imaging modalities particularly nuclear imaging. While the use of NIR imaging aids in the maximum achievable depth of signal penetration, this is still limited when compared with nuclear imaging techniques that demonstrate unlimited depth penetration. Furthermore, OI quantification is challenging due to light attenuation differences experienced in different organs. These challenges impede the use of optical molecular imaging agents in patients.

Thus far, various NIR agents have been developed for use in the imaging of cancer cells, sentinel lymph nodes (SLN), cardiovascular, neurological and inflammatory diseases. Due to limitations brought about by depth penetration and attenuation, the majority of fluorescent agents have been used for the purpose of intraoperative surgery, tumour margin assessment or as contrast agents for hypervascularity. Currently, there are two fluorophores approved by the FDA for clinical use; fluorescein and indocyanine green (ICG) (Figure 1.3). Fluorescein has been successfully implemented for use in ophthalmology, while ICG has been used as a reagent for testing hepatic function. More recently, ICG has been used as a contrast agent in the clinical evaluation of arthritic inflammation by assessing the angiogenesis characteristic of this disease.
1.2 Current imaging techniques for the detection of rheumatoid arthritis

In Canada, the annual cost of rheumatism is estimated at 6 billion Canadian dollars and as such, represents a substantial economic burden as one of the most financially draining diseases. This can largely be attributed to delayed diagnosis resulting in expensive corrective orthopedic procedures. Today, the “accepted” and most frequently prescribed modality used to image the disease remains conventional radiography. Because this imaging technique is the basis of many widely accepted grading systems followed by physicians, diagnosis and monitoring of the disease is restricted to anatomical manifestations that arise in later stages such as bone erosion and joint destruction. This presents a health care predicament, since current medications used to treat RA are only effective at early stages of the disease, rendering prescribed disease-modifying anti-rheumatic drugs (DMARD) ineffective. The bone erosion and joint-space narrowing are well defined in radiography but signify irreversible damage. As a result of the limitations of anatomical methods, there has been a recent interest in being able
to image molecular processes associated with RA\textsuperscript{24} (Table 1.1.). Various studies have shown that early therapeutic intervention resulting from early diagnosis promotes reversal of the molecular events of the disease.\textsuperscript{25}

<table>
<thead>
<tr>
<th>Entry</th>
<th>Class</th>
<th>Radioisotope</th>
<th>Target</th>
<th>Reference</th>
</tr>
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<td>macrophages</td>
<td>26</td>
</tr>
<tr>
<td>2</td>
<td>immunoglobulins</td>
<td>99\textsuperscript{m}Tc, 111\textsuperscript{In}</td>
<td>Fc receptor</td>
<td>27</td>
</tr>
<tr>
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<td>111\textsuperscript{In}</td>
<td>E-selectin</td>
<td>28</td>
</tr>
<tr>
<td>4</td>
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<td>99\textsuperscript{m}Tc</td>
<td>lymphocytes</td>
<td>29,30</td>
</tr>
<tr>
<td>5</td>
<td>monoclonal antibodies</td>
<td>99\textsuperscript{m}Tc</td>
<td>TNF-α</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>peptide (octreotide)</td>
<td>99\textsuperscript{m}Tc, 111\textsuperscript{In}</td>
<td>somatostatin receptor</td>
<td>31,32</td>
</tr>
<tr>
<td>7</td>
<td>glucose analog</td>
<td>18\textsuperscript{F}</td>
<td>metabolizing cells</td>
<td>25</td>
</tr>
</tbody>
</table>

\textbf{Table 1.1} Radiolabeled agents used for the molecular imaging of RA.

Many of the strategies employed for nuclear imaging now have OI surrogates. This is because penetration depth affecting small joints of the hand which are typically involved in the disease are unaffected and can be easily visualized.\textsuperscript{40} Examples include the tagging of monoclonal antibodies and vitamins with fluorophores.\textsuperscript{33} A notable example includes the conjugation of folic acid to a NIR cyanine dye, permitting the targeting of the over-expressed folate receptor in activated macrophages.\textsuperscript{34} Table 1.2 summarizes the various optical techniques used clinically for assessing rheumatic diseases.
Figure 1.4 Scintigraphic images of wrists of a RA patient after injection with $^{99m}\text{Tc}$ labeled adalimumab a) before therapy, 6 hours after administration b) before therapy, 20 hours after administration c) after 3 months of therapy, 6 hours after administration d) after 3 months of therapy, 20 hours after administration.\(^{25}\)

<table>
<thead>
<tr>
<th>Technique</th>
<th>Assessment</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>Non targeted</td>
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<td>25,32,37,40</td>
</tr>
<tr>
<td>Targeted</td>
<td>over-expression of targets</td>
<td>34,38</td>
</tr>
<tr>
<td>Activity-Based*</td>
<td>cathepsin B activity</td>
<td>41</td>
</tr>
</tbody>
</table>

*used in osteoarthritis mice model.

Table 1.2 Different clinical approaches using optical probes in rheumatology.

Non-targeted optical agents have become fast and easy methods to image hypervascularity in RA. In 2008, a non-targeted optical procedure known as “Rheumascan” was clinically approved in Europe to assess changes in microcirculation associated with RA.\(^{32,42,43}\) Indocyanine green (ICG) is injected
intravenously and optical images are obtained based on localization of the dye. This will differ dramatically in a RA patient as a result of the high microvascular density and increased capillary blood flow associated with the disease. In this way, the signal enhancement provided by the localization of the dye is directly proportional to the neoangiogenesis found in the synovial membrane of a RA patient.

1.3 Reactive Oxygen Species (ROS)

The term “reactive oxygen species” (ROS) is used to describe any reactive molecules and radicals derived from the partial reduction of molecular oxygen (Figure 1.5). These molecules are central to all aerobic organisms as they are pivotal players in various biological functions such as signal transduction, immune system control, gene expression, cellular signaling, and neurotransmission.\textsuperscript{44} Redox regulation of ROS is necessary to prevent the over-production of ROS in the cell, which would otherwise trigger the destruction of cellular components such as DNA and cell membranes. This phenomenon, whereby the redox status of the cell is in the pro-oxidative state is often referred to as “oxidative stress” and is found in various disease states including inflammatory diseases\textsuperscript{45}, Alzheimers, Parkinson’s disease\textsuperscript{46} and cancer.\textsuperscript{47} The role of ROS in pathogenesis is not limited to impairment at the macromolecular level. ROS has been heavily linked to gene activation leading to tumour metastasis and in this way, ROS has been associated with both initiation and progression of disease.\textsuperscript{48}
Figure 1.5 Common ROS produced intracellularly.

Under normal conditions, cells possess multiple defense mechanisms to impart a balance in the intracellular redox status, thus preventing the damaging effects of oxidative stress. Superoxide dismutases (SOD) are a class of enzymes that catalyze the conversion of superoxide to hydrogen peroxide and oxygen (eq 1.1). The generated hydrogen peroxide is then converted to water and oxygen by catalase enzymes (eq 1.2). Numerous antioxidants are also found in the cell to promote protection against ROS, such as vitamin C, glutathione and vitamin E.

\[
\begin{align*}
2O_2^- + 2H^+ & \xrightarrow{\text{SOD}} H_2O_2 + O_2 \quad \text{(eq 1.1)} \\
2H_2O_2 & \xrightarrow{\text{Catalase}} 2H_2O + O_2 \quad \text{(eq 1.2)}
\end{align*}
\]

Endogenous ROS are primarily produced in the mitochondria through the electron transport chain (ETC) or by oxidoreductase enzymes. It has been reported that the ETC is responsible for 90% of cellular oxygen consumption.
which 1-5% are converted to ROS under normal physiological conditions. These species are formed as a direct consequence of oxidative phosphorylation, a vital cellular process that consumes oxygen to create adenosine triphosphate (ATP). The ETC carries out oxidative phosphorylation through five proteins, simply known as complex I, II, III, IV and V (Figure 1.5). Electrons are passed through the ETC via electron donors NAD(P)H (taking place at complex I) and FADH$_2$ (taking place at complex II), which ultimately lead to the reduction of oxygen to water at complex IV. This sequence of redox events simultaneously causes protons (H$^+$) to be pumped into the intermembrane space from the mitochondrial matrix, generating a mitochondrial membrane potential ($\Delta \Psi_m$). This generated electrochemical gradient is the main driving force for ATP synthesis in complex V. Leakage of electrons at complex I and complex III can occasionally result in the reduction of molecular oxygen to superoxide (O$_2^-$). 

![Figure 1.6 Schematic representation of the ETC and mitochondrial ROS production.](image-url)
Superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) generation within the mitochondria are widely known and have been studied extensively.$^{50}$ H$_2$O$_2$ is produced via conversion of O$_2^-$ through the enzyme SOD.$^{52,53}$ As a result of its electrophilic character and short half-life, O$_2^-$ is less likely to be found exterior to the mitochondrial membrane or be involved in cellular signaling.$^{51}$ Instead, this ROS may promote generation of other species (such as ONOO$^-$) which stimulate irreversible cellular destruction.$^{54}$ Comparatively, H$_2$O$_2$ is considerably more stable and accordingly intracellular concentrations of H$_2$O$_2$ are 100-fold higher than O$_2^-$, making it more likely to be involved in signaling.$^{55}$ Exceedingly reactive ROS such as the hydroxyl radical (·OH) are produced as secondary products to O$_2^-$ and H$_2$O$_2$ generation.$^{51}$ More specifically, ·OH is produced via Fenton chemistry catalyzed by iron and copper commonly present in the mitochondria. The in vivo half-life of ·OH is said to be $10^{-9}$ seconds and the short-lived nature of this radical makes it extremely destructive to cellular components and therefore, profoundly involved in pathogenesis.$^{56}$

As previously mentioned, various redox enzymes independent of the mitochondria are also responsible for ROS production which themselves further increase ROS concentrations in the mitochondria. This phenomenon is known as “ROS-induced-ROS”.$^{51}$ Such enzymes include NADPH oxidase, xanthine oxidase and (uncoupled) eNOS.$^{51}$ The best known example of “ROS-induced-ROS” is the positive feedback mechanism whereby NADPH oxidase ROS activation results in
angiotensin (ang II) promoted mitochondrial ROS which further promotes NADPH oxidase derived ROS.\textsuperscript{57}

1.3.1. The role of ROS in RA

Activated macrophages play a critical role in RA pathogenesis. The oxidative stress brought about by a high concentration of ROS in arthritic joints has been linked to the localization and function of T-cells.\textsuperscript{45} In response to oxidative stress, macrophages migrate and destroy the surrounding synovial joint.\textsuperscript{45} RA patients exhibit an increase in ROS levels in the inflammatory site and a decreased ability to counteract the imbalance in the redox potential of a cell. Activated macrophages found in the synovial joint compartment display an over expression of the enzyme NADPH oxidase, which further promotes activation of these macrophages.\textsuperscript{45} This redox imbalance provided by the over production of ROS also promotes the increased levels of biological markers, cytokines and the over-expression of various metalloprotease enzymes including the enzyme TNF-\(\alpha\) converting enzyme (TACE).\textsuperscript{58} Various experiments have confirmed that ROS levels govern the behavior of T-cells in the inflammatory site, suggesting that the redox status of the cell is a significant tool to utilize in detection and treatment of the disease.\textsuperscript{59}

1.3.2 Existing probes used to assess elevated ROS levels: challenges and limitations

While some ROS sensing probes function via spin-trap mechanisms (e.g. TEMPO derived probes) \textsuperscript{60} the majority of reported probes in the literature are
based on fluorescence response. Historically, the most commonly used fluorescent ROS probes (available through commercial sources) for *in vitro* cell-based assessment of ROS are dihydroethidium (DHE) and 2,7-dichloro dihydrofluorescein (DCFH) which respond to $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ respectively (Figure 1.7).

![Chemical structures](image)

**Figure 1.7** - a) Conversion of non-fluorescent DHE to fluorescent ethidium (ET, $\lambda_{\text{ex}} = 510 \text{ nm}$, $\lambda_{\text{em}} = 610 \text{nm}$) in response to superoxide b) Conversion of non-fluorescent DCFH to fluorescent 2,7-2,7-dichlorofluorescein (DCF, $\lambda_{\text{ex}} = 498\text{nm}$, $\lambda_{\text{em}} = 522\text{nm}$) c) Conversion of non-fluorescent DHR 123 to rhodamine 123 ($\lambda_{\text{ex}} = 500\text{nm}$, $\lambda_{\text{em}} = 536\text{nm}$).

The rhodamine-derived ROS probe dihydrorhodamine 123 (DHR 123) is also used routinely for the detection of various ROS species including $\text{H}_2\text{O}_2$, $\cdot\text{OH}$ and...
HOCl. These probes are membrane permeable which make them useful in assessing oxidative burst within cells.\textsuperscript{61} Upon O$_2^-$ oxidation, DHE is converted to the fluorescent DNA intercalator ethidium (ET) and the more recently discovered oxidation product hydroxylethidium.\textsuperscript{62} Although this probe is frequently employed as an \textit{in vitro} sensor of ROS, there are numerous limitations and challenges associated with its use that also hold true for other mitochondria-targeted probes such as Mitox-red.

Firstly, DHE and its derivatives spontaneously oxidize creating higher background signals and responses that do not precisely correlate with ROS levels.\textsuperscript{63} This is also a concern with the use of DCFH and DHR 123.\textsuperscript{64} Furthermore, the success of the probe heavily relies on concentrations used in the assay. At higher concentrations of DHE, fluorescence can be seen irrespective of superoxide-induced oxidation and is associated with saturation of mitochondrial nucleic acid binding sites by ET.\textsuperscript{65} Importantly, while it is commonly acknowledged that DHE responds exclusively to O$_2^-$, it must be noted that it is also sensitive to forming multiple oxidation products as a result of H$_2$O$_2$ associated peroxidases and other ROS species.\textsuperscript{66} Chang and coworkers have developed a series of boronate functionalized fluorescein derivatives that are selective for the detection of H$_2$O$_2$ making these derivatives attractive alternatives to DCFH (Figure 1.8b).\textsuperscript{67}

Recently, there has been considerable interest in developing novel ROS probes with distinct structural features that may help address some issues with presently employed probes.\textsuperscript{61} General properties for successful ROS probes include
designing agents that are stable and resistant to auto-oxidation, exhibit minimal cytotoxicity, are membrane permeable and exhibit a drastic change of optical properties upon oxidation so as to minimize background.\textsuperscript{68} If mitochondrial targeting is required, cationic lipophillic species are designed to accumulate within the mitochondrial inner membrane which is driven by the negative potential generated by the proton gradient of the mitochondrial membrane (Figure 1.8a).\textsuperscript{61c}

\textbf{Figure 1.8} a) mitochondria-targeting ROS probe MitoSOX b) boronate probe functionalized fluorescein H$_2$O$_2$ probe.

While considerable progress has been made on the development and design of ROS probes, translation to \textit{in vivo} clinical applications have been challenging. In 2009, Kundu \textit{et al.} developed a series of ROS probes based on the reduction of the iminium cation of commercially available cyanine dyes.\textsuperscript{69} These probes, known as “hydrocyanines”, are a series of membrane permeable non-fluorescent species that upon selective oxidative hydrogen abstraction by O$_2^-$ or ·OH, generate fluorescent cationic species that become retained within the ROS active cell. These probes demonstrated the first preclinical example of ROS detection \textit{in vivo} and since then have been evaluated in a number of ROS models.\textsuperscript{70}
Although technological advancements have significantly improved fluorescence response \textit{in vivo}, fluorescent probes continue to suffer from key limitations in detection and quantification (section 1.4). Consequently, a considerable effort has been made to develop ROS probes that are capable of being detected \textit{via} nuclear imaging techniques which would allow for non-invasive and quantitative imaging that is not limited in depth penetration (Figure 1.10).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{hydrocyanine.png}
\caption{General structure of hydrocyanine ROS probes.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{pet-ros.png}
\caption{PET-ROS probes reported in the literature.}
\end{figure}
Mach and coworkers have developed the first $^{18}$F labeled ROS probe based on a synthesized DHE derivative which was shown to exhibit a two-fold uptake in a ROS cardiac model (Figure 1.10a).\textsuperscript{71} Similarly, Chang and coworkers reported a boronic acid that releases the PET tracer $[^{18}$F]FLT upon exposure to $\text{H}_2\text{O}_2$.\textsuperscript{72} Recently, Okamura et al. have successfully developed a $^{11}$C labeled dihydroquinoline ROS probe capable of crossing the blood-brain barrier.\textsuperscript{73} While these probes have paved the way for a novel approach towards detecting ROS in living systems, the optimal agent has yet to be developed.

1.4 Thesis objectives, overview and rationale

The long-term goal is to develop molecular imaging probes to assess early RA. These probes can be used as a tool to not only detect the disease at earlier stages, but also to monitor response to treatment, which becomes extremely important considering the severe side effects that may accompany current medications. This thesis describes three main approaches towards imaging RA. The first approach involves the development of a multi-modal OI-PET tracer that targets ROS which is found in the synovial tissue of RA patients. Given the persistence of high ROS levels in different diseases, this probe can also be used in the assessment of infection, other inflammatory diseases, cancer and neurodegenerative diseases. The second approach involves developing similarly “non-targeted” fluorophores and/or “turn-on” ROS probes that show improved properties such as efficient delivery, improved solubility and enhanced “turn-on” capabilities. This approach relies on the angiogenesis pathology linked to multiple pathological conditions. The
third approach involves preliminary efforts to create a “targeted” approach involving the development of molecules that could be radiolabeled and specifically target the enzyme TACE which is over-expressed by elevated ROS in the synovium of RA patients.\textsuperscript{74}

Given the dominant role of ROS in disease progression and initiation, molecular imaging of ROS is a highly desirable imaging technique that has yet to be addressed clinically. One reason for the lack of these probes is that they are difficult to access. More importantly, the assessment of ROS pathology via a multi-modal imaging strategy offers complimentary advantages when OI and PET imaging are integrated. Such a combination can impart improved temporal/spatial resolution provided by OI and deep depth penetration provided by PET.\textsuperscript{75} Accordingly, this permits the use of a single agent for various different clinical purposes (\textit{i.e.} the PET labeled agent used for \textit{in vivo} assessments while the “cold” counterpart can be used for image-guided surgeries) and is the main driving force behind the evolution of the successful lymph node draining agent Tilmanocept.\textsuperscript{76}

Fluorine-18 (\textsuperscript{18}F) remains the most commonly employed PET isotope as a result of its valuable nuclear/chemical properties such as its ability to possess the shortest positron range (\( \approx 2.3 \) mm) resulting in superior resolution when compared to other PET isotopes.\textsuperscript{77} For creating ROS probes, the incorporation of fluorine is challenging due to the harsh conditions often involved in the incorporation of \textsuperscript{18}F and the thermal sensitivity of these chromophores.\textsuperscript{78} For these reasons, there are only two examples of \textsuperscript{18}F-labeled NIR cyanine dyes; one of which involves the
synthesis of aryltrifluoroborates and the other of which requires a pre-labeled prosthetic group (described further in Chapter 4). Consequently, before developing an OI-PET ROS probe, several hurdles must be overcome. The specific goals in this context were:

- Development of a straightforward and late-stage $^{18}$F labeling strategy for ROS sensitive dyes in which lengthy syntheses and the use of prosthetic groups can be avoided.
- The synthesis of stable precursors that can be readily separated from labeled products.
- To create a probe that does not require conjugation to a targeting ligand.
- Incorporation of $^{18}$F in a manner that does not afford bulky substituents which could drastically impede pharmacokinetics/pharmacodynamics.
- Develop a labeling approach with short reaction times.
- Development of a fluorophore that activates in diseased tissue thus minimizing background signals of the optical probe.

These goals were achieved through the development of novel OI-PET probes (Figure 1.11). The target ROS probes were prepared (Chapter 2 and Chapter 3) using various *meso*-chlorinated NIR cyanine dyes where ROS sensing ability of the corresponding hydrocyanines was demonstrated by Kundu *et al*. The incorporated cyclohexyl ring provides additional rigidity and offers advantages in comparison to their “open chained” counterparts including enhanced photostability and quantum yields.$^{78}$ The $o$-fluoropyridine functionality was chosen as it is
established that labeling at this position is facile and high-yielding.\textsuperscript{79} Additionally, this functionality is resistant to metabolic defluorination and has been shown to be successfully retained in cancer cells as compared with corresponding fluoroarene derivatives.\textsuperscript{80}

In addition to this approach, there are various other ways to target regions of high ROS pathology including using the “enhanced permeability and retention” (EPR) effect.\textsuperscript{81} This relies on the premise that diseased tissue such as that found in RA joints and tumours, experience angiogenesis. This is the underlying principle behind the “Rheumascan” procedure described in section 1.4. The main disadvantage of this procedure is instability and lipophilicity of the contrast agent (\textit{i.e.} ICG) which can result in aggregates, decreasing fluorescence quantum yields. As a result, a subsequent goal having created new ROS probes was to develop a means to address this issue by encapsulation of the fluorophores in F-127 micelles in an effort to improve optical stability and delivery of the agent to the area of interest (Chapter 3).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{oi-pet-probe.png}
\caption{General structure of OI-PET ROS probe}
\end{figure}
This thesis also describes the synthesis of hydroxamic/hydroxamate TACE inhibitors (Chapter 5) that were modified for the purpose of creating targeted imaging agents for RA.\textsuperscript{82} Targeting TACE for the purpose of molecularly imaging RA is a unique approach and rationale behind this imaging strategy is outlined below:

- TACE has never been exploited as a MI target and over-expression of this enzyme is an event specific to RA in rheumatology and therefore the envisioned probe will be able to distinguish between other rheumatic diseases.
- The ligands chosen as targeting vectors are small molecule inhibitors with high affinity and specificity to TACE. These radiolabeled compounds are more likely to exhibit faster clearance, rapid uptake and retention in the synovium (pharmacokinetic parameters), in comparison to larger antibodies previously used. This can limit prolonged radiation exposure to the patient.

The preliminary work describes the synthesis and \textit{in vitro} screening of these modified compounds through an established fluorogenic assay. With the success of the ROS probes described in Chapters 2, 3 and 4, further work on optimizing these compounds and creating imaging probes for detecting RA in addition to ROS-active tumours is possible and a key goal of future research.
1.5 References


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Chapter 2: A Fluorine-18 Labeled Near-IR Turn-on Dye for Multi-Modal Reactive Oxygen Species (ROS) Detection

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2.1 Preface

The following work contains text and figures that is in preparation for submission to Chemistry-A European Journal. Salma Al-Karmi performed and developed the synthetic work, characterization, purification, optical induction studies and all experiments related to radiolabeling. Dr. Silvia Albu aided in the synthetic work and characterization. Nancy Janzen and Shannon Czorny performed the in vivo and in vitro biological experiments. Laura Banevicius performed preliminary excitation and emission spectra. Dr. Max Nanao and Dr. Jon Zubieta generated crystal structure data. Dr. Alfredo Capretta and Dr. John Valliant are the principle investigators and led the research efforts.

2.2 Introduction

Reactive oxygen species (ROS) are central to all aerobic organisms as they play a pivotal role in signal transduction, immune system control, gene expression and cellular signalling.\(^1\) A homeostatic balance via redox regulation of ROS is required to prevent a pro-oxidative cellular state; a phenomenon known as “oxidative stress.” Extensive oxidative destruction brought about by oxidative stress has been implicated in the initiation and progression of multiple diseases
including Alzheimer’s and Parkinson’s disease, cancer, and inflammatory diseases. Consequently, the ability to detect elevated ROS levels in vivo has significant value for basic research, drug discovery and patient care.

The majority of ROS sensing probes reported to date are based on fluorescence response. To overcome limited depth of imaging that is achievable using optical techniques, progress in preparing radiolabeled ROS probes has been made recently. For example Mach and co-workers reported the synthesis of \(^{18}\text{F} \) labeled analogue of the widely used in vitro ROS sensing dye dihydroethidium (DHE). More recently, Chang and co-workers reported a novel construct that releases the PET tracer \([^{18}\text{F}]\text{FLT} \) upon exposure to hydrogen peroxide.

2.3 Objectives

While these probes have a number of attractive features, there is a notable absence of a single construct that can be used for both PET and near-infrared (NIR) imaging of ROS. NIR probes offer superior depth penetration and reduced light scattering compared to other fluorophores. Fortuitously, the reduction of the iminium cation of NIR cyanine dyes has been shown to create sensitive ROS probes, which react via oxidative hydrogen abstraction by superoxide and hydroxyl radicals. Reaction with these species “turns-on” the fluorescence and generates a cationic fluorophore that remains in ROS active cells. While select groups have reported \(^{18}\text{F} \) labeling of near-IR dyes, we sought to develop a convenient route to an \(^{18}\text{F}-\)labeled, near-IR turn-on type ROS probe. The approach takes advantage
of an inexpensive cyanine dye as the starting material and a late-stage fluorination, to ensure ease of access and use for in vitro and in vivo ROS studies.

2.4 Synthesis, Radiolabeling and Biological Evaluation

To synthesize the target probe, the commercially available dye IR780 iodide was functionalized via a Suzuki coupling with 6-fluoropyridin-3-ylboronic acid (Scheme 2.1).\textsuperscript{14-18} \textit{o}-Fluoro-pyridine was selected as the prosthetic group, because it is known to be resistant to metabolic defluorination and the \textsuperscript{18}F analogue can be produced from the corresponding nitropyridine.\textsuperscript{19} Radiopharmaceuticals derived from fluoro-pyridines have also shown enhanced uptake and retention in cancer cells compared to analogues derived from fluorobenzene.\textsuperscript{20}

![Scheme 2.1 Synthesis of the fluorinated ROS probe 1a from a commercially available IR780 dye.](image)

Following isolation of 2, the dye was reduced with sodium borohydride in methanol to afford the ROS probe 1a in 82% yield. Reduction of the iminium bond was immediately accompanied by a change in colour (green to yellow). Absorption and emission spectra revealed that 2 exhibited excitation and emission maxima at 768 nm and 794 nm respectively, with a Stokes shift of 26 nm, which is comparable
Excitation of 1a at 768 nm showed no fluorescence in direct contrast to 2 (Figure 1). This drastic change in intensity has been shown to be advantageous where turn-on type probes reduce background signal and maximize signal-to-noise ratios for both in vitro and in vivo studies.21

Figure 2.1 Fluorescence emission spectra for 1a and 2 (40 μM in methanol, \( \lambda_{\text{ex}} = 768 \) nm).

DHE, a commonly employed in vitro ROS probe,11 requires careful handling as it is prone to auto-oxidation.22 A comparison of the stability profile of 1a with DHE in 20% methanol/PBS buffer (pH=7.4) was performed by placing 40 μM solutions of the probes in a water bath at 37°C, and taking fluorescence measurements over time. The experiments showed that 1a was stable to auto-oxidation, while DHE experienced a steady increase in fluorescence intensity over 24 h.
Hydrocyanine-based ROS probes have been shown to respond selectively to hydroxyl radicals and superoxide. Using the same assay, the reactivity of 1a towards a series of different ROS sources and related reagents was evaluated to assess any potential impact of adding the fluorinated prosthetic group. Fluorescence emission at 794 nm (λ<sub>ex</sub> = 768 nm) was triggered upon treatment with hydroxyl radicals and superoxide (Figure 2.2), which is consistent with other hydrocyanine ROS probes. For the other ROS sources, along with Fe (II) and glutathione (GSH), the emission intensity was similar to the vehicle control. To assess the relative change in intensity upon exposure to ROS, 1a, and DHE were treated with potassium superoxide (KO<sub>2</sub>), and the fluorescence monitored after 1 h. Compound 1a demonstrated a much greater response than DHE, and there was significantly less background signal for the hydrocyanine in the absence of the radical source. The fluorescence enhancement for 1a was 21 fold, while for DHE it was less than 2 (1.53).

Fluorescence microscopy studies using PC-3 cells, which are known to generate high concentrations of ROS, were performed using 1a. The images obtained indicated the dye localized in the cells and that the fluorescence intensity was reduced when the cells were treated with ROS quenchers, NAC and TEMPOL (Appendix 1, Figure S12). Notwithstanding, having demonstrated uptake and retention in PC-3 cells, the cellular toxicity was evaluated noting that 1a appeared to well tolerated during all microscopy studies. Using an MTT assay, even after incubation for 48 hr, the IC<sub>50</sub> (65 μM) was higher than that for the cisplatin control.
Figure 2.2 Fluorescence response of 1a (20 μM in 9% methanol/PBS) to various oxidants: a) OtBu b) ·OH c) ClO− d) O2 e) O2 f) Fe2+ g) H2O2 h) GSH (λex = 768 nm, λem = 794 nm).

To prepare a suitable precursor for labeling with 18F, a Suzuki coupling with IR780 iodide and 2-nitro-5-pyridineboronic acid pinacol ester 3 (Scheme 2.2) was performed. The product was obtained in 68% yield and the crystal structure of 3 determined after the product was crystallized from an ethanol/water mixture (Appendix 1, Figure S14 and Table S1). The crystal structure was consistent with that proposed for 3, and showed a fully conjugated backbone where the average sp2 C-C bond lengths were 1.37 Å (± 0.04). The data is in agreement with previously reported X-ray structures of cyanine dyes.24,25
Scheme 2.2 Synthesis of 2-nitropyridines 3 and 4 as precursors for labeling with $^{18}$F.

$^{18}$F labeling (to produce 1b) was initially performed by subjecting 3 to standard nucleophilic fluorination conditions followed by treatment with methanolic borohydride. Despite attempts to optimize the method, the highest decay corrected radiochemical yield (DCY) obtained was 19%. A more successful route involved direct labeling of 4 at 150 °C for 4 min, which increased the DCY to 62% ($n = 5$), and further decreased the overall time required for synthesis and purification by approximately 60 min (Scheme 2.3). It is important to note that this approach required strict time control, as prolonged exposure to borohydride resulted in drastically reduced labeling yields.

Scheme 2.3 One-step $^{18}$F radiolabeling of 4 to produce 1b.
Compound 1b was purified by reverse phase HPLC and dried under reduced pressure prior to formulation where the total synthesis/purification time was 35 min. The identity of 1b was confirmed by coinjection with the non-radioactive standard 1a (Figure 2.3). The specific activity was calculated using a standard curve and was found to be 5.2 GBq/µmol (0.14 Ci/µmol), which is consistent with the modest amount of starting activity used (0.92 GBq). The value is comparable to previously reported \(^{18}\)F BODIPY fluorophores,\(^{8c,8d,8h,9a}\) but lower than trifluoroborate based dyes\(^{8e,9b}\) and \(^{18}\)F o-pyridine based radiopharmaceuticals, where the latter indicates it is feasible to achieve higher specific activities.\(^ {26}\)

![Figure 2.3](image)

**Figure 2.3** UV (top) and gamma HPLC traces of a coinjection of non-radioactive standard 1a and radiolabeled product 1b.

The stability of 1b in 10% ethanol/saline was assessed using HPLC. At 60 min, 1b remained the dominant product however over time a small peak in the HPLC, corresponding to the retention time of the oxidized fluorophore, was evident. As this is likely due to the presence of radicals generated following radioactive
decay, the use of a radical scavenger was explored. Ascorbic acid, at concentrations suitable for preclinical and clinical studies (0.5 mg/mL), was used as an antioxidant and proved to be highly effective as the probe showed no evidence of oxidation out to 150 min (Appendix 1, Figures S19 and S20).

The biodistribution of cyanine dyes including IR780 using fluorescent imaging has been evaluated in a range of different preclinical models. With access to $[^{18}\text{F}]1\text{b}$, it is possible to use PET imaging to evaluate the in vivo distribution of the reduced hydrocyanine dye. PET/CT images at 2 and 4 h showed significant blood pool activity where the heart, lungs and spleen could be readily visualized (Figure 2.4).

**Figure 2.4.** PET/CT image (left) of 1b at 2 h post-injection (PI) (75% max. threshold) and biodistribution data (right) at 2 and 4 h PI, expressed as percent injected dose per gram (%ID/g). Complete biodistribution data and additional images are found in Appendix 1; Table S2, Figure S22.
Quantitative tissue and fluid counting following necropsy was in agreement with the images and confirmed that at 2 h the highest amount of activity was in the blood (52.3 ± 4.6 %ID/g) followed by spleen (23.6 ± 1.4 %ID/g) and gall bladder (17.4 ± 3.4 %ID/g), with activity also present in blood rich organs (heart, lungs, liver). The observed retention of the tracer in the blood pool may be due to binding of 1b to albumin or red blood cells (RBC’s, Chapter 3).29,30

To assess the ROS targeting ability of the probe \textit{in vivo}, 1b was evaluated in a ROS-induced cardiomyopathy model whereby C57BL/6 mice were administered doxorubicin (Dox, 20 mg/ Kg). We observed a significant increase in heart uptake between Dox-treated and control mice at 4 hours post injection of the tracer ($p < 0.05$, Figure 2.5).

![Figure 2.5](image)

**Figure 2.5** Percent injected dose per gram (%ID/g) in the heart of untreated and doxorubicin treated C57BL/6 mice 4 hours post injection of 1b ($n = 3$, $p < 0.05$).

Compound 1b exhibited a smaller change as compared to [\textsuperscript{18}F]DHE (1.3 versus 2-fold), which may be attributed to the selectivity of 1b towards hydroxyl
radicals and superoxide whereas DHE is known to react with a wider range of ROS and reactive nitrogen species\textsuperscript{31} that are present in Dox-induced cardiomyopathy models.\textsuperscript{32,33}

2.4 Summary and Conclusions

In summary, we report a convenient synthetic method for preparation of a $^{18}$F-labeled multi-modal PET-turn-on NIR ROS probe. The fluorophore 1\textit{a} is synthetically tractable and has a number of advantages over existing ROS sensing probes including its ability to emit in the NIR region upon selective oxidation and is resistant to autooxidation. The PET analogue 1\textit{b} can be produced in a single step in reasonable radiochemical yield and exhibits stability in solution. This probe was tested in a doxorubicin cardiac model and demonstrated significant cardiac uptake as compared with untreated mice The work describes the first reported PET and quantitative biodistribution study of a radiolabeled hydrocyanine dye that shows selective \textit{in vitro} and \textit{in vivo} reactivity towards ROS.

2.5 Experimental

Supplementary figures can be found in \textbf{APPENDIX 1}.

\textbf{General considerations.} Unless otherwise stated, all chemicals and reagents were purchased and used as received from Sigma-Aldrich without further purification. Solvents were purchased from Caledon Laboratories Ltd. Reactions were monitored using Alugram Sil G/UV\textsubscript{254} thin layer chromatography (TLC) plates
and visualized under ultra-violet light. Column chromatography was performed on a Teledyne Isco Combiflash Rf200® system.

$^1$H and $^{13}$C NMR spectra were recorded on Bruker AV 700 or AV 600 spectrometers. $^1$H chemical shifts are reported in ppm, relative to the residual proton signal of the NMR solvents. Coupling constants ($J$) are reported in Hertz (Hz). $^{13}$C chemical shifts are reported in ppm, relative to the carbon signal of the NMR solvents. High resolution mass spectra (HRMS) were obtained on a Waters QToF Ultima Global spectrometer. Reverse phase analytical HPLC was performed using a Waters 2489 HPLC, equipped with a Waters 2489 UV/Vis ($\lambda = 254 \text{ nm or 350 nm}$) and a Bioscan Flow count gamma detector (model 106). Spectra were recorded and processed on Empower 2 software (Waters). The eluents were HPLC grade water containing 0.4% ammonium formate (solvent A) and acetonitrile (solvent B). Analytical HPLC was performed using a Phenomenex Synergi Polar RP column (250 × 4.60 mm, 4 µm). Semi-preparative HPLC was performed using a Phenomenex Synergi Polar RP column (250 × 10.0 mm, 4µm). The flow rate for analytical HPLC was 1 mL/min and 4 mL/min for semi-prep HPLC. The desired products were analyzed/eluted using either a 95 to 5% gradient (solvent A) over 35 min (method A) or a 50% to 5% gradient (solvent A) over 25 min (method B). Fluorescence measurements were obtained using a Tecan® infinity M1000 plate reader while microscopy studies were performed using Olympus Bright Field BX 53 microscope. Cell media and supplements were purchased from Invitrogen (Mississauga, ON), and the PC-3 cell line purchased from ATCC (CRL-1435). Cells
were propagated using Ham’s F-12 Nutrient Mix, supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin Streptomycin at 37°C and 5% CO₂ and were not used beyond passage 30. Fluorine-18 was produced using GE-PET trace-10 cyclotron. 18F fluoride was trapped on a QMA cartridge preconditioned with 0.2% KHCO₃ and sterile water solution and was eluted off the QMA cartridge using in 2 mL 0.04 mmol/mL K222 solution.

**Synthesis of 2**

IR780 iodide (25 mg, 0.04 mmol), 6-fluoropyridin-3-ylboronic acid (7.0 mg, 0.05 mmol), Pd(OAc)₂ (1.0 mg, 10 mol%) and t-BuXPhos (3.0 mg, 20 mol%), were added to a vial equipped with a Teflon® cap and purged under argon for 2 minutes. The contents of the vial were suspended in ethanol (1 mL) and argon bubbled through the solution for 5 min using a long needle. K₃PO₄ (aq) (0.5 mL, 0.2 mM) was added under argon and the vial sealed, covered with aluminum foil and heated at 100°C overnight. The reaction vessel was cooled and the reaction mixture diluted with water (10 mL) and extracted with DCM (2 × 10 mL). The organic phases were combined, dried over anhydrous MgSO₄ and concentrated in vacuo. The desired product was isolated using a 8 gram flash RediSep® Rf basic alumina column (using 10% MeOH in DCM as the eluent) to afford 2 as a green solid (19 mg, 79%). Rᵣ = 0.26 (5% MeOH/DCM). ¹H NMR (700 MHz, MeOH-d₄): δ 8.12 (d, J = 2.4 Hz, 1H), 7.90 (dt, J = 7.9, 2.4 Hz, 2H), 7.41-7.36 (m, 5H), 7.27 (d, J = 7.9 Hz, 2H), 7.21 (dt, J = 7.5, 0.6 Hz, 2H), 7.15 (d, J = 14.0 Hz, 2H), 6.25 (d, J = 14.0 Hz, 2H), 4.08 (t, J = 7.4 Hz, 4H), 2.75 (t, J = 6.1 Hz, 4H), 2.07 (m, 2H), 1.84 (dd, J
= 14.7 Hz, 7.4 Hz, 4H), 1.84 (m, 4H), 1.27 (s, 12H), 1.02 (t, J = 7.4 Hz, 6H). $^{13}$C NMR (175 MHz, MeOH-d4) δ 173.7, 165.0 ($J = 241.1$ Hz), 157.2, 149.1 ($J = 14.1$ Hz), 148.3, 145.0 ($J = 8.0$ Hz), 144.0 ($J = 273.3$ Hz) 134.5 ($J = 4.7$ Hz), 133.3, 129.8, 126.3, 123.5, 112.1, 111.0 ($J = 37.1$ Hz), 101.6, 50.1, 49.9, 46.5, 28.0, 27.9, 25.8, 22.4, 21.8, 11.6; HRMS (ESI⁺) for C$_{41}$H$_{47}$N$_3$F calcd 600.3754, obsd 600.3760.

**Synthesis of 1a**

Compound 2 (10 mg, 0.016 mmol) was dissolved in MeOH (2 mL) in a 30 mL scintillation vial covered with aluminum foil. Freshly prepared NaBH$_4$ in MeOH (1 mL, 0.16 mM) was added dropwise to the green solution, generating a yellow solution. The reaction was stirred at room temperature (RT) for 10 min and the solvent was removed by rotary evaporation. The resulting solid was dissolved in DCM (3 mL) and extracted with water (2 × 3 mL). The organic phase was dried over anhydrous MgSO$_4$ and the solvent was removed by rotary evaporation to afford a bright yellow solid (7.0 mg, 82%). $R_f = 0.31$ (hexanes). $^1$H NMR (600 MHz, CD$_3$CN): δ 7.92 (s, 1H), 7.64 (d, $J = 7.1$ Hz, 1H), 7.13 (m, 1H), 7.09 (dd, $J = 8.0$ Hz $J = 1.0$ Hz, 2H), 7.06 (d, $J = 7.3$ Hz, 2H), 6.98 (dd, $J = 7.7$ Hz, $J = 1.0$ Hz, 2H), 6.95 (d, $J = 7.2$ Hz, 2H), 6.74 (appt, $J = 7.4$ Hz, 2H), 6.64 (d, $J = 8.0$ Hz, 2H), 6.58 (appt, $J = 7.4$ Hz, 2H), 6.41 (d, $J = 7.7$ Hz, 2H), 6.04 (d, $J = 15.6$ Hz, 1H), 5.90 (d, $J = 12.4$ Hz, 1H), 5.70 (dd, $J = 15.6$ Hz, $J = 9.0$ Hz, 1H), 5.49 (d, $J = 12.4$ Hz, 1H), 3.56 (t, $J = 7.3$ Hz, 2H), 2.90 (m, 2H), 2.58 (m, 2H), 2.52 (m, 2H), 1.98 (m, 2H), 1.64 (m, 2H), 1.11 (s, 9H), 0.96 (s, 3H), 0.93 (t, $J = 7.4$ Hz, 3H) 0.85 (t, $J = 7.4$ Hz, 3H). δ $^{13}$C NMR (150 MHz, CD$_3$CN) δ 150.4 ($J = 269.2$ Hz), 149.4 ($J = 13.6$ Hz), -44-
HRMS (ESI$^+$) for C$_{41}$H$_{49}$N$_3$F calcd 602.3911, obsd 602.3916

**Synthesis of 3**

IR780 iodide (25.0 mg, 0.04 mmol), 2-nitro-5-pyridineboronic acid pinacol ester (12 mg, 0.05 mmol), Pd(OAc)$_2$ (1.0 mg, 10 mol %) and $t$-BuXPhos (3.0 mg, 20 mol %) were added to a vial equipped with a Teflon® cap and purged under argon for 2 min. The contents of the vial were suspended in absolute ethanol (1 mL) and argon bubbled through the solution for 5 min using a long needle. K$_3$PO$_4$ (aq) (0.5 mL, 0.2 mM) was added under argon and the vial sealed, covered with aluminum foil and heated at 100°C overnight. The reaction vessel was cooled and the reaction mixture diluted with water (10 mL) and extracted with DCM ($2 \times 10$ mL). The organic phases were combined, dried over anhydrous MgSO$_4$ and concentrated in vacuo. The product was isolated using a 8 gram flash RediSep® Rf basic alumina column (using 20% MeOH in DCM as the eluent) to afford 4 as a green solid (17 mg, 68%). $R_f = 0.52$ (5 % MeOH/ DCM). $^1$H NMR (700 MHz, MeOH-d$_4$: $\delta$ 8.52 (s, 1H), 8.51 (d, $J = 5.4$ Hz, 1H), 8.07 (dd, $J = 8.2$ Hz, 1.8 Hz, 1H), 7.36 (t, $J = 7.7$ Hz, 2H), 7.32 (d, $J = 7.3$ Hz, 2H), 7.20 (m, 4H), 6.91 (d, $J = 14.0$ Hz, 2H), 6.19 (d, $J = 14.0$ Hz, 2H), 3.99 (t, $J = 7.3$ Hz, 4H), 2.73 (t, $J = 6.1$ Hz, 4H), 2.13 (s, 6H), 2.03 (m, 4H), 1.79 (m, 4H), 1.16 (d $J = 8.1$ Hz, 12H), 0.99 (t, $J = 7.4$ Hz, 6H). $^{13}$C NMR (175 MHz, MeOH-d$_4$: $\delta$ 173.5, 157.6, 155.1, 150.2, 147.5, 143.5, 142.9, 142.5,
Auto-oxidation study

A 40 µM solution (1 mL) of 1a (in 20% MeOH/PBS buffer, pH = 7.4) was placed in a water bath at 37°C and the fluorescence intensity readings ($\lambda_{ex}/\lambda_{em} = 768$ nm/794 nm) of 100 µL aliquots were taken every h for 7 h and once at 24 h. This was repeated for 40 µM solution (1 mL) of dihydroethidium (DHE, $\lambda_{ex}/\lambda_{em} = 515$ nm/560 nm) for comparison (Figure S9).

Stability studies for 1a

A 30 µM solution of 1a in 20% MeOH/PBS (1 mL) was incubated at 37°C for 6 h. The solution was analyzed by analytical HPLC after 1 h and 6 h (Figure S10).

Reactivity assay for 1a

Analytes (500 µM, final concentration) were added to a solution of 1a (final concentration: 20 µM) in 9% MeOH/PBS buffer (pH =7.4) and left at ambient temperature for 1 h, after which fluorescence was measured using a plate reader ($\lambda_{ex} = 768$ nm, $\lambda_{em} = 794$ nm). KO$_2$ was added as a solid while hydroxyl radicals were generated in situ via the Fenton reaction where H$_2$O$_2$ (100 mM) was added to an aqueous solution of iron (II) sulfate heptahydrate (100 mM). tert-Butoxyl radical was generated by combining tert-butyl hydrogen peroxide (TBHP,100 mM) with Fe$^{2+}$ (100 mM). Singlet oxygen ($^1$O$_2$) was generated in situ by the addition of NaClO$_4$ to H$_2$O$_2$.36
Reaction with superoxide

To a solution of 1a (10.0 mg, 0.016 mmol) in 10 mL dry DMSO was added 10 mg of solid KO$_2$. The reaction was stirred for 1 h at RT. The stock solution was used to make a 50 μM solution in MeOH and 150 μL of this solution was used for fluorescence measurements (λ$_{ex}$/λ$_{em}$ = 768 nm/794 nm). The readings were recorded relative to a 150 μL of 50 μM solution of untreated 1a (Figure S11). The experiment was repeated for (10.0 mg, 0.016 mmol) DHE (λ$_{ex}$/λ$_{em}$ = 510 nm/ 595 nm).

Fluorescence microscopy studies

PC-3 cells (2.5 × 10$^5$ cells/well) were plated on 12 mm round coverslips and incubated with or without 5 mM NAC in Ham’s F12 nutrient mixture (Invitrogen, 11765-054), containing 10% FBS and 1% penicillin/streptomycin at 37°C and 5% CO$_2$ for 24 h. Cells were also pre-treated with or without 5 mM TEMPO$_\text{L}$ for 30 min or 5 mM NAC for 1 h, prior to addition of 1a. Test compound was added directly to the growth media and incubated for 30 min at 37°C. Cells were washed twice with PBS, and fixed by incubating in 4% paraformaldehyde for 10 min at RT. Following fixation, cells were washed twice with PBS and once with H$_2$O. Coverslips were mounted to slides using Everbrite Hardset Mounting Medium containing DAPI, and stored at 4 °C overnight. The cellular fluorescence was acquired on an Olympus Bright field BX53 microscope using a commercially available filter set consisting of a 747 nm excitation filter, and a 800 nm emission filter (Appendix 1, Figure S12)
MTT assay

PC-3 cell viability and proliferation was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Molecular Probes, V13154). PC-3 cells were plated (1.0x10^4 cells/100µL) in growth media and incubated at 37°C under 5% CO₂ atmosphere overnight. 1a was suspended in 4% DMSO/Ham’s F12 Nutrient Mixture buffer and a serial dilution was performed to achieve final concentrations/well of 0.01, 0.1, 1, 10, 100 and 1000µM in triplicate. As a control, cisplatin was added to wells at identical concentrations and conditions. No cell as well as vehicle only controls were also completed in triplicate. The cells were incubated for 48 h at 37°C under 5% CO₂ atmosphere. The media was aspirated off and replaced with fresh growth media containing no phenol red (Invitrogen, 31053). MTT was added (1mM final concentration in each well) and incubated at 37°C for 4 h, after which 100 µL of SDS-0.01M HCl (1 g/mL) was added to each well. The cells were incubated at 37°C overnight and the absorbance was recorded at λ = 570 nm. (Appendix 1, Figure S13)

X-ray crystallography

Data were collected on the ESRF microfocus MX beamline ID23-EH. A crystal of 3 was flash frozen in a gaseous Nitrogen stream at 100K, and oscillation data were collected on a Pilatus3-2M detector (Dectris, Switzerland) at 14.209 keV (0.8726 A). The detector was set to the minimum possible distance, which is limited by the diffractometer and sample changer robotics to 1.06 A at the edge. Higher resolution
data can however be collected at the corners of the detectors. No 2θ movement exits on this beamline, and the energy is fixed. 1480, 0.15° oscillations were collected, with 0.04 second exposures, and 99% attenuation of the beam. Data were reduced with XD.\textsuperscript{38}

Structure refinement was carried out using the SHELXTL crystallographic software.\textsuperscript{39} After assigning all non-hydrogen atoms, the models were refined against F\textsuperscript{2} first using isotropic and then using anisotropic thermal displacement parameters. The hydrogen atoms were introduced in calculated positions and then refined isotropically. Neutral atom scattering coefficients along with anomalous dispersion corrections were taken from the International Tables, Vol. C. Images of the crystal structures were generated using CrystalMaker.\textsuperscript{40}

**Synthesis of 1b**

Compound 3 (5.5 mg, 0.007 mmol) was dissolved in 400 µL of MeOH, and 200 µL of a freshly prepared 0.03 mM sodium borohydride solution in MeOH was added slowly. An instant colour change from green to yellow was observed. The reaction was stirred at RT for 30 s after which it was extracted with DCM (2 × 10 mL). The organic phase was dried over anhydrous MgSO\textsubscript{4} and the solvent was removed by rotary evaporation to afford the reduced product as a yellow-brown solid (4.9 mg). A solution of K [\textsuperscript{18}F] F-K\textsubscript{222} complex (0.92 GBq) was transferred to a 5 mL pyrex\textsuperscript{®} reaction vial and dried at 85°C in an oil bath under a stream of nitrogen. Azeotropic drying by the addition of 1 mL portions of CH\textsubscript{3}CN was performed over a 20 minute
period (total of 6 mL). The reduced precursor was dissolved in 400 µL of dry DMF and added to the $^{18}\text{F/K}_{222}$ mixture which was subsequently heated at 150°C for 4 minutes. After cooling to RT the desired product was isolated by semi-preparative HPLC (decay corrected RCY = 62% ± 5, $n = 5$).

For imaging and biodistribution studies, following evaporation under reduced pressure $1b$ was formulated in 10% ethanol/2% pluronic (F-127) in 0.9% sterile saline.

**Stability study for 1b**

Compound $1b$ (19.98 MBq) was suspended in 600 µL of 10% ethanol/saline and incubated at RT. The solution was analyzed by reverse-phase HPLC (Method A) at 60 and 120 minutes (Appendix 1, Figure S19).

Stability was also accessed in the presence of ascorbic acid (Appendix 1, Figure S21). Compound $1b$ (21.98 MBq) was suspended in 500 µL of 10% ethanol/saline. To this was added 100 µL of a 0.5 mg/mL aqueous solution of ascorbic acid. The mixture was left to incubate at RT and the solution was analyzed by reverse-phase HPLC at 60 and 150 minutes (Method A, Figure S20).

**Specific activity of 1b**

Compound $1b$ (2.85 MBq) was dried and allowed to decay to background. It was then dissolved in MeOH and the fluorescence intensity at ($\lambda_{ex}$ 418 nm) measured and compared to a standard curve (Figure S21) determined using known
concentrations of 1a (30 μM, 15 μM, 7.5 μM, 3.7 μM, 1.8 μM, 938 nM, 469 nM, 234 nM). The specific activity was 5.18 GBq/µmol (0.14 Ci/µmol).

**In vivo experiments**

All animal studies were approved by the Animal Research Ethics Board at McMaster University. Mice were maintained under Specific Pathogen Free (SPF) conditions in an established animal facility with 12 h light/dark cycles and given food and water libitum. Imaging experiments were performed on healthy female 17-18 week old C57BL/6 mice (Taconic, Germantown, NY, USA). Biodistribution experiments were performed on healthy female 5-6 week old C57BL/6 mice (Charles River Laboratories, Kingston, NY, USA).

**PET/CT imaging**

Imaging experiments were performed on a healthy female C57BL/6 mouse, 17-18 weeks old. Each mouse was injected with approximately 9.7MBq of the radiotracer 1b (200μL in 2% pluronic F127/0.9% NaCl) via the tail vein. For the PET imaging, mice were anesthetized with 1% isoflurane and strapped to a bed equipped with an electric heating coil (Harvard). The bed is sealed inside of a Biocontainment Cylinder, and a plastic tube with two airlines, each fitted with a filter (PALL Gas Filter with Hydrophobic Glass, 8004022), allowed continuous infusion of anesthetic into the chamber. The cylinder is placed into the gantry of the MOSAIC Animal PET Imaging System (Philips) such that the animal is positioned within the field of view of the scanner. A 60 min list mode acquisition was initiated immediately after
injection of the radioactive tracer. Post acquisition, the data was sorted into twelve 5 min images, which were reconstructed with non-attenuation correction and standardized uptake value (SUV) conversion. At later time points, 15 min static acquisitions were acquired. Sinograms were reconstructed with non-attenuation correction and SUV conversion. For the CT images mice were anesthetized using 1% isoflurane and X-ray images acquired using a conebeam X-SPECT scanner (Gamma Medica, Nothridge, USA), with a source voltage of 75 kVp and a current of 165 $\mu$A at the McMaster Centre of Preclinical and Translational Imaging. Projection data was acquired with 1024 projection angles (1184x1120 pixels, 0.1 mm pixels) and reconstructed using a Feldkamp cone beam backprojection algorithm in COBRA (Exxim Software, Pleasanton, CA, USA), into 512x512x512 arrays (0.155 mm isotropic voxels). A water-filled tube was included within each scan in order to convert the voxel values to Hounsfield units (HU). CT images were compressed to a 256x256x256 matrix (0.31 mm isotropic voxels) then the CT and PET images were fused, with in-house software developed in Matlab, by a process that maximised mutual information (MI) between the two images. Powell's multidimensional direction set method was used to maximise MI using a one-dimensional search algorithm based on golden section search and parabolic interpolation. PET images underwent rigid body transformation until a change of less than 0.01 mm (translation) or 0.01 degrees (rotation) was observed along or around each axis to obtain fusion parameters. During the process, the 823 matrix of the PET image was interpolated for comparison to the CT image, and was
resampled to a $256^3$ matrix when the stop criteria were met. This corresponds to a change in voxel size from $1.463 \text{ mm}^3$ to $0.31 \text{ mm}^3$. When maximized MI was reached, the result was visually inspected to confirm fusion quality. Imaging analysis was completed using AMIDE software. (Appendix 1, Figure S22)

**Biodistribution studies**

Biodistribution studies were performed on healthy female C57BL/6 mice, 5-6 weeks old ($n = 3$ per time point, at $t = 2\text{h}$ and $4\text{h}$ post-injection; an additional mouse remained under anesthetic for $1\text{h}$ post-injection). Mice were injected with approximately $0.7\text{MBq}$ of $1b$ (200µL in 2% pluronic F127/0.9% NaCl) via the tail vein. At the specified timepoints, animals were anesthetized with 3% isoflurane and euthanized by cervical dislocation. Blood, adipose, adrenals, bone (femur), brain, gall bladder, heart, kidneys, large intestine and caecum (with contents), liver, lungs, pancreas, skeletal muscle, small intestine (with contents), spleen, stomach (with contents), thyroid/trachea, urinary bladder + urine 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). (Appendix 1, Table S2)
2.6 References


39. SHELXTL PC, version 6.12, Bruker-AXS Inc., Madison, WI, **2002**


Chapter 3: Biological Studies on First Generation Fluorinated-near-IR Probes

3.1 Introduction

The biological properties of probes 1a/1b and 2a/2b (Chapter 2) were further investigated including assessing differences in their distribution in vivo. The high blood residence time of 1b led to speculation that the agent may be binding to red blood cells (RBCs) or serum albumin and as such, may be useful as a blood-pool agent capable of assessing hypervascularity. Such an agent can be valuable in evaluating inflammatory diseases (such as RA), cancer, HIV and lymph-node draining.

Cyanine dyes such as ICG and other fluorescent dyes such as Evans blue have been known to bind serum albumin. It is this feature that aids in the delivery of the agents to the area of interest. Further advantages of albumin binding agents include improved pharmacokinetics and circulation, and prevention of premature clearance of the probe. ICG itself has been known to show enhanced fluorescence while bound to albumin in comparison to the free dye. Despite these advantages, the intravenous use of ICG is limited in part by the instability of the fluorophore in solution. Accordingly, ICG forms multiple degradation products both in vitro and in vivo which promotes rapid excretion by the liver and bile duct.

Radiolabelled ligands for the assessment of blood pool volume have been sought after in an effort to address limitations associated with fluorescence imaging. Clinically, 99mTc labeling of RBCs is one of the most common ways of
assessing blood pool activity. However, this method suffers from poor image quality and requires drawing the patient’s blood prior to administration, which present risks, both to the patient and healthcare provider. To improve image quality, many attempts have been made to create PET blood pool agents. This includes $^{11}$C or $^{15}$O labeling of RBCs via gaseous carbon monoxide, which requires elaborate instrumentation due to the route of administration involving inhalation by the patient. Albumin labeling is an attractive alternative to RBC mediated blood volume imaging. Currently, the only albumin-derived radiopharmaceutical approved by the FDA is $^{131/125}$I-labelled HSA. Preclinical PET imaging has since been performed on $^{68}$Ga, $^{62}$Cu, $^{64}$Cu and $^{18}$F labelled HSA. Notwithstanding, many HSA radiopharmaceuticals require pre-conjugation of a labeled prosthetic group to the protein prior to administration. Other concerns include in vivo stability of the complex, less than ideal circulation and high concentration of the tracer in the liver or lungs, which are known to possess a large albumin space.

Another approach used to extend the half-life of a probe and improve its targeting efficiency is the encapsulation of the agent in a micelle. This approach is especially useful for hydrophobic optical probes as it has been shown to improve in vivo stability, hydrophilicity and clearance. Specifically, encapsulation of cyanine dyes into micelles and nanoparticles have been shown to increase quantum yield by 450-fold in an aqueous environment and an increase of circulatory half life by 95-fold. These agents can accumulate in pathological tissue experiencing angiogenesis via the EPR effect where retention of these
probes are as a consequence of poor lymphatic drainage at pathological sites.\textsuperscript{20, 21} This strategy can therefore improve delivery, while also enhancing \textit{in vivo} stability, half-life and uptake.\textsuperscript{18}

Additionally, such probes can be used in theranostics and offer an opportunity to precisely target diseased tissue.\textsuperscript{21} NIR fluorophores (such as ICG) amplify heat generation within targeted tissue and generate cytotoxic heat upon NIR excitation.\textsuperscript{22} Thus cyanine dyes (such as ICG, IR780 and IR783) and other NIR probes have been encapsulated in micelles and successfully employed as photosensitizers in photothermal therapy (PTT) and photoacoustic imaging (PAI) in addition to acting as improved NIR imaging agents.\textsuperscript{19, 23-28}

\textbf{3.2 Objectives}

This chapter describes studies investigating two complementary approaches in imaging angiogenesis. First, as an extension of Chapter 2, a comparison is made in the \textit{in vivo} biodistribution and imaging data of PET agents 1\textit{b} and 2\textit{b} in healthy C57BL/6 mice. This study will provide valuable information on whether the biological behaviour of the probes is comparable and whether the presence of the iminium cation is necessary for blood retention of the tracer. Furthermore, a comparison of these studies will determine whether tracer 1\textit{b} undergoes oxidation \textit{in vivo} and thus provides valuable insight on the stability of the probe. Additional \textit{in vivo} experiments where the blood of the animal is analyzed will determine whether the probe resides in the blood plasma or bound to RBCs.
In the second approach, encapsulation of synthesized NIR fluorinated fluorophores in pluronic F-127 micelles is discussed. Anticipated advantages of this approach include improved solubility in aqueous solutions, enhanced photochemical properties and improved in vivo half-life. Furthermore, many cyanine dyes are plagued by problems associated with toxicity and micelle encapsulated dyes are known to decrease or even prevent these toxic effects.\textsuperscript{18} Therefore, potentially higher concentrations of these micelles can effectively be used in a theranostic approach for imaging and treatment of diseases possessing hypervascularity. Pluronic F-127 polymers have been approved by the FDA and exhibit well-documented safety profiles. Successful F-127 encapsulated agents can therefore be translated to human use.\textsuperscript{29}

### 3.3 Biodistribution, Imaging and Blood Binding Studies of PET tracers 1b and 2b

Compounds 1b and 2b were radiolabeled directly as described in Chapter 2. Due to their hydrophobicity, these tracers exhibited poor solubility and transfer efficiency which resulted in problematic i.v. administration in mice. For this reason, it was necessary to explore various biocompatible formulations prior to any in vivo studies (Table 3.1). The most successful formulation (entry 12) was also used for tracer 2b, but this agent demonstrated considerably less transfer efficiency than 1b (˂ 8\%). This difference in behaviour may be attributed to attraction of the cation to the negatively charged surface of the glass vial. To validate this, SigmaCote® was used to neutralize the surface of the glass prior to formulation which resulted in an increase in transfer efficiency (≈ 30\%). The addition of ethanol to the
formulation was necessary as it was later found that this not only aided in transfer of the agent but also improved the stability of the compound, which in its absence would degrade due to radiolysis (Figure 3.1).

<table>
<thead>
<tr>
<th>Entry</th>
<th>Buffer</th>
<th>Additive</th>
<th>% Transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>PBS</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>Saline</td>
<td>10% EtOH</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Saline</td>
<td>5% BSA</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>Saline</td>
<td>10% PEG-400</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>Saline</td>
<td>10% CD</td>
<td>12</td>
</tr>
<tr>
<td>7</td>
<td>Saline</td>
<td>5% BSA</td>
<td>25</td>
</tr>
<tr>
<td>8</td>
<td>Saline</td>
<td>0.01% Tween</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>PBS</td>
<td>10% ETOH, 10% CD</td>
<td>13</td>
</tr>
<tr>
<td>10</td>
<td>PBS</td>
<td>10% ETOH, 10% PEG 400</td>
<td>7</td>
</tr>
<tr>
<td>11</td>
<td>PBS</td>
<td>10% ETOH, 1% DMSO</td>
<td>12</td>
</tr>
<tr>
<td>12</td>
<td>Saline</td>
<td>2% F-127, 10% EtOH</td>
<td>50</td>
</tr>
<tr>
<td>13</td>
<td>Saline</td>
<td>2% F-127</td>
<td>44</td>
</tr>
</tbody>
</table>

Table 3.1 Various biocompatible formulations explored for 1b and corresponding transfer efficiencies. CD = β-cyclodextrin. % transfer is defined as (initial radioactivity in vial / final radioactivity measured after transfer) × 100.

Biodistribution studies performed on 1b showed high retention of the tracer in the blood out to 4 hours (Table 3.2, 53% and 42% ID/g at 2 and 4 hours respectively). Organs possessing a large blood volume such as the spleen and liver showed high uptake which remained relatively constant over the duration of the study. The high uptake in the gall bladder suggests the tracer is being cleared via the hepatobiliary system. The modest activity in the skeleton implies some defluorination of the tracer, but is quite surprising given that ortho-fluoropyridines are known to be quite resilient in vivo.\textsuperscript{30} PET/CT images confirmed the
biodistribution data where the tracer was retained in the circulatory system for the duration of the study and blood-rich organs especially ventricles of the heart were clearly visualized.

![Graph](image)

**Figure 3.1** γ-Traces of 1b a) after 0.5 hours formulated in 10% ethanol, 2% pluronic F-127 in saline b) formulated in 2% pluronic F-127/saline 1-hour post formulation c) formulated in 2% pluronic F-127/saline 2 hours post formulation.

The biodistribution pattern of 1b bears a remarkable resemblance to existing blood-pool agents, especially those related to serum albumin. For example, studies on $^{125}$I labeled albumin demonstrated considerable uptake in organs with high albumin accumulation namely the adrenals, spleen, heart and liver with obvious high retention in the blood.\textsuperscript{31}
The long circulation time of the tracer suggests that the compound is bound to the blood tightly and that the resulting complex is stable. This feature is highly desirable in a blood pool agent as many agents tend to suffer from stability issues resulting in rapid clearance.\textsuperscript{13,32} For example, \textsuperscript{18}F and \textsuperscript{11}C labeled RBCs showed 23-53\% decrease in blood pool activity after 60 minutes.\textsuperscript{33} Another example involving \textsuperscript{67}Cu labeled albumin showed complete clearance of the tracer from the

Table 3.2 Biodistribution of 1b after intravenous injection into healthy C57BL/6 mice, sacrificed at 2 and 4 h. Data is expressed in % injected dose/gram of tissue or fluid (%ID/g).

<table>
<thead>
<tr>
<th>Organ/Tissue</th>
<th>%ID/g 2h n=3 Avg</th>
<th>%ID/g 2h n=3 SEM</th>
<th>%ID/g 4h n=3 Avg</th>
<th>%ID/g 4h n=3 SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>52.32 4.57</td>
<td>42.51 10.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adipose</td>
<td>0.72 0.07</td>
<td>1.02 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenals</td>
<td>13.25 2.04</td>
<td>12.29 2.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone</td>
<td>3.7 0.36</td>
<td>4.73 1.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>0.76 0.06</td>
<td>0.59 0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gall Bladder</td>
<td>17.45 3.45</td>
<td>11.16 1.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>5.14 0.38</td>
<td>4.39 1.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidneys</td>
<td>9.41 2.86</td>
<td>7.46 2.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lg Intestine + Caecum</td>
<td>1.33 0.25</td>
<td>3.87 0.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>7.98 0.31</td>
<td>10.21 1.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lungs</td>
<td>9.89 1.03</td>
<td>7.44 1.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>1.59 0.12</td>
<td>1.13 0.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>0.72 0.05</td>
<td>1.17 0.67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sm Intestine</td>
<td>2.27 0.1</td>
<td>1.95 0.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>23.63 1.36</td>
<td>24.37 4.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>0.92 0.15</td>
<td>1.5 0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroid/Trachea</td>
<td>3.24 0.47</td>
<td>3.22 0.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine + Bladder</td>
<td>1.5 0.57</td>
<td>2.2 1.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
blood in just 10 minutes.\textsuperscript{15} Given the lipophilicity of 1b (log P \approx 10), it may be possible that the agent is preferentially binding to the hydrophobic pocket (in site IIA) of albumin which is dominated by strong hydrophobic interactions.\textsuperscript{34} Thorarensen \textit{et al.} \textsuperscript{35} developed a very high affinity fluorescent albumin binder that possesses common functionalities to 1b (Figure 3.2). In fact, the binding of indole containing compounds to albumin are well-documented and the literature notes various examples.\textsuperscript{36}

![Figure 3.2](image)

\textbf{Figure 3.2} Common structural features between 1b and potent HSA binder 6 (400 \pm 100 nm).

To determine definitively whether the tracer resided in the blood plasma or RBCs, blood studies were performed where the tracer was injected in mice and the blood collected. Whole blood was centrifuged and activity in the supernatant and RBC were counted (Figure 3.3). This experiment showed that 97\% of the activity was found in the blood plasma and not RBC’s, further supporting that the tracer may be binding to serum albumin.
Figure 3.3 Percent activity for compound 1b in red blood cells and plasma post centrifugation of perfused blood.

To rule out the possibility that the added surfactant may have promoted the observed prolonged circulation, biodistribution studies and PET/CT imaging were performed on 2b using the same formulation (Figure 3.4 and 3.5). The distribution of radioactivity in tissues drastically varied from that seen with 1b. At 2 hours, an average of 7.51% ID/g was seen in the blood and blood pool activity decreased to 0.6% ID/g at the 4-hour time point. Comparatively less activity was found in blood rich and albumin-accumulating organs such as the adrenals, the spleen and the liver. At 4 hours, higher amounts of activity were detected in the gall bladder and large intestine suggesting the tracer was clearing via the hepatobiliary system. This finding differs from 1b which showed very little clearance from the blood and only slight variation in organ distribution between 2 and 4-hour time points. This data is
in agreement with the PET/CT image which highlights the gallbladder and large intestine as the predominant visualized organs (Figure 3.5).

![Figure 3.4 Biodistribution for 2b after intravenous injection into healthy C57BL/6 mice, sacrificed at 2 and 4 h. Data is expressed as percent injected dose per gram of tissue or fluid (%ID/g).](image)

This data suggests that 2b either does not bind to components of the blood or does so very weakly as compared to 1b, though it has been previously noted that the related dye IR780 exhibits affinity to albumin.\(^{38}\) It is strongly inferred through this data that the presence of the iminium ion is responsible for this change in \textit{in vivo} behaviour. This study was repeated with 2b and it was shown that any activity observed in the blood similarly resided in the blood plasma (data not shown). In comparison to 1b (and in agreement with the \textit{in vivo} studies) much less activity
was observed in the blood for 2b, further demonstrating that this tracer does not bind as strongly to components in the blood plasma. In agreement with the above data, the carcass showed appreciatively less activity than those that received 1b further confirming that 2b clears at a sufficiently higher rate.

**Figure 3.5** Reconstructed PET/CT image at 2 hours post intravenous injection of 2b in a healthy C57BL/6 mouse.

To confirm that the compound is capable of binding albumin, fluorescence studies were performed on 1a. Binding of fluorophores to albumin is typically accompanied by an increase in fluorescence intensity, indicative of the dye being enclosed by a non-polar environment.\textsuperscript{39} 1a at a final concentration of 20 μM, was incubated with various concentrations of HSA. The compound exhibited an enhancement of fluorescence intensity with increasing concentrations of HSA, suggestive of a binding interaction between 1b and HSA (Figure 3.6). Further, at
higher concentrations (not shown for clarity), no observable difference in fluorescence intensity was observed, indicating saturation of binding sites on the protein.\textsuperscript{39}

\textbf{Figure 3.6} Fluorescence intensity spectra upon incubation of 1a ($\lambda_{\text{ex}} = 412$ nm, 20 $\mu$M final concentration) with varying concentrations of HSA (0-4.5 $\mu$M). Some HSA concentrations were omitted from the plot for clarity.

3.4. \textit{The synthesis, characterization and assessment of encapsulated NIR fluorinated dyes in pluronic F-127 micelles}

Synthesized NIR dyes 2 and 7 were encapsulated in micelles using F-127 pluronic coblock polymers. IR780 iodide was also encapsulated to serve as a reference standard for \textit{in vivo} imaging studies. This reference was chosen for several reasons. Firstly, IR780 iodide as a free dye has been known to accumulate in tumours.\textsuperscript{40} This implies that upon dissociation from the micelles, uptake into the
tumour can be observed. Secondly, lipophilic fluorophores, such as IR780 have previously been bound to nanocarriers and shown promise in both imaging and PTT/PAI/PDT therapy.\textsuperscript{21,38} Importantly, IR780 loaded nanoparticles have also been shown to exhibit stronger intensity in tumours than the free unbound dye.\textsuperscript{38} Studies involving IR780 encapsulated micelles led to the development of nanoimaging agent LipImage\textsuperscript{TM}, where the encapsulated dye is similar in structure to IR780.\textsuperscript{26} Thirdly, our synthesized dyes emit within the same range as IR780 but exhibit enhanced fluorescence intensity in comparison (described in Chapter 4).

In addition to the encapsulation of the NIR dyes, ROS probe 1a was also encapsulated with the ultimate goal of improving the aqueous solubility of the probe which proved to be problematic in the \textit{in vitro / in vivo} studies. As, previously mentioned, the micelles can behave as an EPR mediated delivery system. Once delivered to the area of interest, the encapsulated probe (1a) could be released, potentially promoting the oxidation of 1a to 2a in areas experiencing elevated ROS. Hydrocyanine functionalized pluronic F-127 micelles have been utilized previously with great success.\textsuperscript{13}

\textbf{Figure 3.7} Structures of dyes encapsulated in F-127 pluronic micelles.
The micelle encapsulation of the dyes were carried out using a solvent evaporation method as reported by Sreejith et al.\textsuperscript{24} and subsequently purified by dialysis. This method generated monodisperse micelles as shown by experimental PDI values ($\leq 0.2$) determined by DLS. The average hydrodynamic micelle diameter was also determined at $37^\circ$C to be between 19-32 nm for all encapsulated dyes (Figure 3.8).

![DLS data showing hydrodynamic diameter in PBS buffer (pH = 7.4) for a) 1a b) 2a c) 7 and d) IR780.](image)

**Figure 3.8** DLS data showing hydrodynamic diameter in PBS buffer (pH = 7.4) for a) 1a b) 2a c) 7 and d) IR780.

Determination of the diameter by intensity and volume resulted in consistent average diameters. TEM images revealed uniform spherical structures with comparable diameters as those determined by DLS (Figure 3.9). The size of the micelles are very dependent on the preparation procedure and are usually governed by the organic solvent that is used, the addition sequence and concentration of polymer used.\textsuperscript{41}
Figure 3.9 TEM images of a) 1a b) 2 c) 7 and d) IR780 encapsulated micelles in PBS (pH= 7.4).

The average diameter of the micelles are in agreement with some previously reported fluorophore encapsulated F-127 micelles\textsuperscript{23,42} but sufficiently smaller than other fluorophore-encapsulated micelles reported in the literature.\textsuperscript{24} Micelle size is an important feature for optimizing distribution \textsuperscript{41,43} where several studies have shown that nanoparticles >100 nm demonstrate inadequate penetration and accumulation in hypovascular tumours.\textsuperscript{44,45} In their study of drug encapsulated micelles, Cabral \textit{et al.} have shown a significant trend between therapeutic targeting and the relative size of polymeric micelles. Micelles that were 30 nm demonstrated complete tumour suppression, those that were 50 nm showed reduced tumour growth and that 100 nm in size failed to suppress tumour growth.\textsuperscript{43} The inconsistency can be explained by the fact that smaller micelles < 50 nm are capable of traveling to distant blood vessels, permitting homogenous drug
distribution, while larger micelles are unable to efficiently penetrate the tumour. Differences are also seen in accumulation in non-target organs, where larger micelles accumulated more in the liver and non-target organs.\textsuperscript{22,24,43} Additionally, micelles possessing diameters between 10-100 nm are less likely to be prematurely cleared by the reticuloendothelial system, permitting an enhanced EPR effect.\textsuperscript{46}

The entrapment efficiency (EE) and drug loading (DL) were assessed by lyophilizing samples and releasing encapsulated fluorophores by dissolving in a known volume of organic solvent (DMSO or methanol). Absorbance readings were taken and compared to a standard curve fitted to a linear regression model. EE and DL were calculated based on equations 3.1 and 3.2. Table 3.3 summarizes DL, EE and various other properties associated with the micelles.

\[
DL (\text{wt \%}) = \frac{\text{mass of encapsulated fluorophore}}{\text{mass of fluorophores loaded into micelles}} \times 100 \quad (3.1)
\]

\[
EE (\%) = \frac{\text{mass of fluorophore loaded into micelles}}{\text{mass of fluorophore initially added}} \times 100 \quad (3.2)
\]

As shown, the EE and DL efficiencies were quite low. Smaller diameter size and lower EE/DL have been shown to be a factor of higher concentrations of polymer used in the preparation of the micelles.\textsuperscript{23} The method of preparation of these micelles was adapted from Sreejith \textit{et al.} and no EE and DL values were reported for comparison. Low DLs and EEs like the ones obtained have previously been
reported and can be increased by modifying the micelle encapsulation procedure. These low values can also be linked with the high lipophilicities of these fluorophores which is known to influence encapsulation efficacy.

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>PDI</th>
<th>Average Size (nm)</th>
<th>EE (%)</th>
<th>DL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>ND</td>
<td>37.7</td>
<td>40</td>
<td>2.4</td>
</tr>
<tr>
<td>2</td>
<td>0.12</td>
<td>26.6</td>
<td>1.1</td>
<td>0.02</td>
</tr>
<tr>
<td>7</td>
<td>0.24</td>
<td>30.8</td>
<td>21.6</td>
<td>0.43</td>
</tr>
<tr>
<td>IR780</td>
<td>0.25</td>
<td>28.4</td>
<td>8.5</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Table 3.3 Average micelle diameters, % EE, % DL and PDI values for fluorophore encapsulated micelles (ND = not determined).

Micelle stability was initially assessed with samples that were left in PBS at 4°C for 12 days. Specifically, DLS was used to evaluate whether aggregates or changes in diameter could be observed. The micelles showed no changes in PDI and hydrodynamic diameter and no aggregation was observed. Dispersed micelles in PBS were also frozen overnight at -78°C and thawed to perform DLS. Similarly, the micelles showed no change in these properties and aggregation was not observed.

The optical properties of the NIR fluorophore encapsulated micelles were assessed and compared with that of the free dyes. As expected, a red shift in excitation and emission maxima was observed signifying the presence of encapsulated micelles. The fluorescence spectrum of the encapsulated micelles (in PBS) and the corresponding free dyes (in 7% DMSO/PBS) is shown in Figure 3.10. The free fluorophores exhibit fluorescence quenching in aqueous solution, most likely due to aggregation of the dyes.
Figure 3.10 Fluorescence emission scan for encapsulated NIR fluorophores (in PBS) and free dyes (in 7% DMSO/PBS) at a concentration of 14.5 μM. a) emission spectrum of 2 (λ<sub>ex</sub> = 770 nm) b) emission spectrum of 7(λ<sub>ex</sub> = 754 nm) c) emission spectrum of IR780 (λ<sub>ex</sub> = 776 nm).

The stability of the micelles were further evaluated in the presence of BSA (Figure 3.11). The micelles were incubated in 5 % BSA in PBS at 37°C for 1 hour and an emission spectrum was recorded. As a comparison, micelles suspended in PBS in the absence of BSA were also incubated at 37°C. While emission maxima remained the same as for micelles that were not incubated with BSA, the fluorescence intensity was significantly enhanced (Figure 3.11). Kim et al also observed this variation in intensity when their ICG encapsulated F-127 micelles were incubated with serum albumin. Micelles constructed from non-ionic polymers (such as pluronic) have been shown to exhibit stability to blood components and have previously demonstrated favourable biodistribution patterns. Generally, micelles composed of a PEG shell prevent recognition by the immune system and offer prolonged blood circulation necessary for efficient tumour accumulation.
Figure 3.11 Fluorescence emission scan for encapsulated NIR fluorophores incubated at 37°C in the presence and absence of 5% BSA a) emission spectrum of 2 (λ_ex = 770 nm) b) emission spectrum of 7 (λ_ex = 754 nm) c) emission spectrum of IR780 (λ_ex = 776 nm).

It is unclear what led to the change in fluorescence intensity since both micelle stability and albumin bound fluorophores result in enhanced quantum yields. ICG itself (in addition to various other albumin-binding dyes) demonstrates enhanced fluorescence intensity when bound to albumin.\textsuperscript{49} It has also been reported that PEGylated polymers directly interact with serum albumin via hydrogen bonds.\textsuperscript{50} These interactions need not be destructive to the micelles as many have reported micelle stability in the presence of BSA for several hours.\textsuperscript{51} It may be possible that interactions that occur between the polymer and albumin result in enhanced stabilization of the micelle-fluorophore assembly.

In Chapter 2, the toxicity of 1a was assessed and found to have a comparable IC_{50} to cisplatin. Micelle encapsulation has been known to significantly decrease the cytotoxicity of therapeutic agents, such as doxorubicin.\textsuperscript{18b} To evaluate toxicity, an MTT assay was performed on 1a encapsulated in micelles where the average IC_{50} was found to be 2.4 mM (Figure 3.12). In comparison, the parent compound
exhibited an IC$_{50}$ of 52.0 µM, which was comparable to that of cisplatin (65.1 µM). Taken together, the data suggests that the encapsulated probe exhibits considerably less cytotoxicity as compared to the parent compound.

Figure 3.12. MTT assay of PC-3 cell viability, with various concentrations of F-127 encapsulated 1a, cisplatin and blank (empty) micelles.

To evaluate the in vivo properties of the micelles, preliminary studies of encapsulated 1a were performed in a CD1 mouse bearing a PC-3 xenograft tumour. The mouse was injected with 100 µM of the dye and imaged 24 hours post injection. The mouse was imaged at two different wavelengths ($\lambda_{ex} / \lambda_{em} = 418$ nm/478 nm for 1a and $\lambda_{ex} / \lambda_{em} = 768$ nm/794 nm for 2) followed by ex vivo imaging of organs at those same wavelengths (Figure 3.13). The in vivo images obtained show no evidence of accumulation at the tumour and ex vivo imaging confirmed
this at both wavelengths. Under NIR imaging, the liver, kidneys and intestines were the major organs detected.

Figure 3.13. *in vivo* and *ex vivo* IVIS images 24 hours post 10 μg injection of 1a encapsulated micelles a) *in vivo* image at $\lambda_{ex}/\lambda_{em} = 418 \text{ nm}/478 \text{ nm}$ b) *ex vivo* image at $\lambda_{ex}/\lambda_{em} = 418 \text{ nm}/478 \text{ nm}$. c) *in vivo* image at $\lambda_{ex}/\lambda_{em} = 768 \text{ nm}/794 \text{ nm}$ d) *ex vivo* image at $\lambda_{ex}/\lambda_{em} = 768 \text{ nm}/794 \text{ nm}$.

At $\lambda_{ex} / \lambda_{em} = 418 \text{ nm}/478 \text{ nm}$, the major organ visualized was the stomach. Although some fluorescence was detected in the tumour at this wavelength, this signal was not above levels observed in the skin which served as background. One potential reason for the lack of tumour uptake is the prolonged age and large size of the tumour. Larger tumours behave very differently from growing, active tumours.
as they are known to possess decreased rates of cell proliferation and down regulation of apoptosis. Further, PC-3 tumours possess low vascularity and thus assessment of this probe in other highly vascular tumours is worthwhile.

3.5 Summary and Conclusions

This chapter focused on assessing the biological properties of the ROS probe described in Chapter 2. A biodistribution study with the oxidized form suggests that the reduction of the iminium bond is responsible for the strong retention of the tracer in the blood. Blood studies on the ROS probe demonstrate that a substantial amount of the activity in the blood resides in the blood plasma (97%). Furthermore, preliminary fluorescence measurements indicate that 1a may bind albumin.

This chapter also describes the encapsulation of three cyanine fluorophores and a hydrocyanine dye in F-127 pluronic micelles. These micelles proved to be stable 2 weeks in solution and showed significantly higher fluorescence than the corresponding “free” dyes in aqueous solution. When incubated with albumin, the micelles show enhanced fluorescence intensity, which may be due to stabilization of the micelles, or disassembly of the micelle and subsequent interaction of the free dye with albumin.
3.6 Experimental

PET/CT imaging

Imaging experiments were performed using a 18 week old healthy female C57BL/6 mouse. The mouse was injected with approximately 1.7 MBq of the radiotracer 2b (200μL in 2% pluronic F127/0.9% NaCl) via the tail vein. For PET imaging, mice were anesthetized with 1% isoflurane and strapped to a bed equipped with an electric heating coil (Harvard). The bed was sealed inside of a Biocontainment Cylinder, and a plastic tube with two airlines, each fitted with a filter (PALL Gas Filter with Hydrophobic Glass, 8004022), which allowed continuous infusion of anesthetic into the chamber. The cylinder was placed into the gantry of the MOSAIC Animal PET Imaging System (Philips) such that the animal is positioned within the field of view of the scanner. A 60 min list mode acquisition was initiated immediately after injection of the radioactive tracer. Post acquisition, the data was sorted into twelve 5 min images, which were reconstructed with non-attenuation correction and standardized uptake value (SUV) conversion. At later time points, 15 min static acquisitions were acquired. Sinograms were reconstructed with non-attenuation correction and SUV conversion. For the CT images, mice were anesthetized using 1% isoflurane and X-ray images acquired using a conebeam X-SPECT scanner (Gamma Medica, Nothridge, USA), with a source voltage of 75 kVp and a current of 165 μA at the McMaster Centre of Preclinical and Translational Imaging. Projection data was acquired with 1024 projection angles (1184 x 1120
pixels, 0.1 mm pixels) and reconstructed using a Feldkamp cone beam backprojection algorithm in COBRA (Exxim Software, Pleasanton, CA, USA), into 512 x 512 x 512 arrays (0.155 mm isotropic voxels). A water-filled tube was included within each scan in order to convert the voxel values to Hounsfield units (HU). CT images were compressed to a 256x256x256 matrix (0.31 mm isotropic voxels) then the CT and PET images were fused, with in-house software developed in Matlab, by a process that maximised mutual information (MI) between the two images. Powell’s multidimensional direction set method was used to maximise MI using a one dimensional search algorithm based on golden section search and parabolic interpolation. PET images underwent rigid body transformation until a change of less than 0.01 mm (translation) or 0.01 degrees (rotation) was observed along or around each axis to obtain fusion parameters. During the process, the 823 matrix of the PET image was interpolated for comparison to the CT image, and was resampled to a 2563 matrix when the stop criteria were met. This corresponds to a change in voxel size from 1.463 mm³ to 0.31 mm³. When maximized MI was reached, the result was visually inspected to confirm fusion quality. Imaging analysis was completed using AMIDE software.

**Biodistribution studies**

Biodistribution studies were performed on 5-6 weeks old healthy female C57BL/6 mice, \( n = 3 \) per time point, at \( t = 2h \) and \( 4h \) post-injection; an additional mouse remained under anesthetic for 1h post-injection). Mice were injected with approximately 0.7MBq of \( 2b \) (200μL in 2% pluronic F127/0.9% NaCl) via the tail
At the specified timepoints, animals were anesthetized with 3% isoflurane and euthanized by cervical dislocation. Blood, adipose, adrenals, bone (femur), brain, gall bladder, heart, kidneys, large intestine and caecum (with contents), liver, lungs, pancreas, skeletal muscle, small intestine (with contents), spleen, stomach (with contents), thyroid/trachea, urinary bladder, urine 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).

**Blood studies**

Blood studies were performed using healthy female 5-6 week old C57BL/6 mice, (n = 3 per time point, at t = 2h and 4h). Mice were injected with approximately 0.7MBq of 1b (200μL in 2% pluronic F127/0.9% NaCl) via the tail vein. 1mL of blood from each mouse was collected via cardiac puncture while under anesthetic and the total radioactivity was recorded using a dose calibrator. 0.9% NaCl was added to the blood in a 3:1 ratio and the sample was triturated with 0.9% NaCl. Samples were then centrifuged at 600g for 10 minutes. The supernatant was collected and the radioactivity of each fraction was counted in a Perkin Elmer Wizard 1470 Automatic Gamma Counter. Pellets were resuspended in 0.9% NaCl, triturated and centrifuged as before. The remaining radioactivity in the supernatant was counted and added to the previous supernatant wash. Data was expressed as % total radioactivity in the blood sample.
General method for micelle encapsulation of the fluorophores

A solvent evaporation method was performed as described previously. The dye (5 mg) was weighed and dissolved in a 100 mg/mL solution of pluronic F-127 polymer in DCM (total volume: 5 mL). An equal volume of PBS buffer (pH = 7.4) was added to the organic solvent and the mixture was stirred overnight in a 50 mL uncapped round bottom flask. The following day, any remaining DCM was evaporated and the aqueous solvent containing the dispersed micelles was filtered through a 0.2 µm syringe filter. The aqueous mixture was dialysed (Fisherbrand® cellulose dialysis tubing, nominal MW 12,000-14,000) against water for 24 hours.

Determination of loading efficiency and content

Lyophilized samples were weighed and suspended in 1 mL DMSO to release encapsulated fluorophores. Micelles containing no fluorophores were used as a blank. The concentration of the fluorophores was determined using a standard curve. Samples were excited at 780, 766 and 794 nm for dyes 2, 7 and IR780 respectively. Absorbance readings were used to determine concentration from the equation of a line where the squared correlation coefficient were 0.999, 0.998 and 0.997 for 2, 7 and IR780 respectively. The concentrations used ranged from 0.2-30 µM. From this data, % DL and % EE were determined.
Figure 3.14 Standard curve of absorbance versus concentration of 2 in DMSO.

Figure 3.15 Standard curve of absorbance versus concentration of 7 in DMSO.
Figure 3.16 Standard curve of absorbance versus concentration of IR780 in DMSO.

Micelle size determination

Average hydrodynamic diameters and PDI were determined by DLS (Malvern Zetasizer Nano ZS). 1 mg/ mL solutions in PBS buffer (pH = 7.4) were used for the analysis. The readings were taken at 37°C and PDI were reported. Zeta potentials could not be reported due to incompatibility of the probe with the pluronic suspension. Assessment of aggregation and changes in diameter size was assessed after being suspended in PBS (pH = 7.4) for 8 or 12 days at 4°C.

TEM image acquisition

A droplet of a diluted sample (≈ 10 μM) in PBS buffer was placed onto a copper grid (300 mesh) and air dried. The sample was analyzed using a FEI Titan 80-300 LB microscope and average diameters were determined on Image J™ software.
Fluorescence and absorbance measurements

Emission and excitation spectra of encapsulated fluorophores were measured at a concentration of 14.5 µM in PBS buffer. Micelles containing no fluorophores were used as a blank. All optical readings were taken by aliquoting 100 µL of the solutions into a 96-well plate and read using a Tecan® infinity M1000 plate reader.

Stability studies

Micelles at a concentration of 7.25 µM were suspended in 5% BSA/PBS or in the absence of BSA (total volume: 2 mL). The solutions were incubated in a water bath at 37°C for 1 hour. Samples were excited at 780, 766 and 794 nm for dyes 2, 7 and IR780 respectively and the emission spectrum was obtained. PBS or empty micelles in PBS were used as the blank.

MTT assay

PC-3 cell viability and proliferation was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Molecular Probes, V13154). PC-3 cells were plated (1.0x10^4 cells/100µL) in growth media and incubated at 37°C under 5% CO2 atmosphere overnight. 1a was suspended in 4% DMSO/Ham’s F12 Nutrient Mixture buffer and a serial dilution was performed to achieve final concentrations/well of 0.01, 0.1, 1, 10, 100 and 1000 µM in triplicate. As a control, cisplatin was added to wells at identical concentrations and conditions. No cell as well as vehicle only controls were also completed in triplicate. The cells were incubated for 48 h at 37°C under 5% CO2 atmosphere. The media was aspirated
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off and replaced with fresh growth media containing no phenol red (Invitrogen, 31053). MTT was added (1 mM final concentration in each well) and incubated at 37°C for 4 h, after which 100μL of SDS-0.01M HCl (1g/mL) was added to each well. The cells were incubated at 37°C overnight and the absorbance was recorded at λ = 570 nm.

**In vivo and ex vivo fluorescence imaging**

A male CD1 mouse bearing a subcutaneous PC-3 xenograph tumour was injected with 10 μg (in a volume of 200 μL) of 1a encapsulated F-127 micelles in PBS. The mouse was imaged (λ_{ex} / λ_{em} = 418 nm/ 478 nm and λ_{ex} / λ_{em} = 768 nm/ 794 nm) on a IVIS Lumina II at 24 hours and immediately euthanized by cervical dislocation under 2% isofluorane anesthesia. Major organs and the tumour were extracted and imaged on an IVIS system.

3.7 References


Chapter 4: Synthesis of Second Generation Fluorinated Near-Infrared Dyes

4.1 Introduction

The partition coefficient (log P) of a given pharmaceutical is a strong predictor of a compound’s in vivo pharmacokinetic/pharmacodynamics (PK/PD) properties.\textsuperscript{1} This coefficient can effectively correlate with a drug’s absorption, distribution, metabolism and excretion (ADME) properties.\textsuperscript{2} In the case of radiopharmaceuticals, which are generally administered via intravenous injection, endothelial absorption properties are less relevant. Instead, the processes of distribution, metabolism and excretion are of critical importance. Generally, compounds that are lipophilic demonstrate higher plasma binding and therefore, greater overall distribution.\textsuperscript{2} Similarly, lipophilic compounds more readily diffuse through the cell membrane, which is especially important for agents that are required to bind to intracellular targets.\textsuperscript{2}

In addition to exhibiting higher permeability, lipophilic agents tend to be rapidly metabolized by the liver but exhibit slow renal excretion due to passive tubular reabsorption in the kidneys.\textsuperscript{3} Poor renal excretion is especially amplified with compounds that are bound to the plasma as a result of inefficient glomerular filtration.\textsuperscript{3} Radiopharmaceuticals that are too lipophilic are also more likely to exhibit non-specific binding.\textsuperscript{4} Thus it is often very difficult to define an “optimal” log P value.
Generally, the incorporation of fluorine in pharmaceuticals has been known to improve therapeutic efficacy and allow for favourable PK/PD properties. Additional advantages include the high polarity of the carbon-fluorine bond which contributes to its strength (estimated at 130 Kcal/mol) and thus its relative in vivo stability. Although fluorine is often used to replace hydrogen in biomolecules, fluorine is sterically similar to the hydroxyl group and can effectively replace this functionality. Hydrogen and hydroxyl substitutions have afforded clinically relevant radiotracers best exemplified by the development of $[^{18}\text{F}]$ DOPA and $[^{18}\text{F}]$ FDG.

Besides the number of advantages associated with the incorporation of fluorine onto bioactive molecules, a number of fluorinated fluorophores (more specifically, rhodamine and fluorescein derivatives) have been known to possess enhanced optical properties over their non-fluorinated counterparts. For example, the development of a fluorinated fluorescein analogue led to the commercialization of “Pennsylvania Green,” a brighter and more photostable derivative (Figure 4.1).

![Figure 4.1](image)

**Figure 4.1** Fluorinated fluorescein derivatives and their non-fluorinated counterparts.
Similarly, Wurthner et al have shown that the synthesis of fluorinated perylene bisimides accessed via halogen exchange (HALEX), resulted in analogues possessing superior quantum yields in comparison to the corresponding chlorinated and brominated dyes (Scheme 4.1).  

![Scheme 4.1 HALEX reaction performed on chlorinated perylene bidimide producing fluorinated dye 2.](image)

**Scheme 4.1** HALEX reaction performed on chlorinated perylene bidimide producing fluorinated dye 2.

![Figure 4.2 Fluorinated cyanine dyes reported in the literature a) fluorination via alkylation at the indole nitrogen b) fluorinations at the aryl ring position c) fluorination at the cyclohexyl position has not been reported.](image)

**Figure 4.2** Fluorinated cyanine dyes reported in the literature a) fluorination via alkylation at the indole nitrogen b) fluorinations at the aryl ring position c) fluorination at the cyclohexyl position has not been reported.

Fluorination of fluorophores have been limited in number, mainly due to difficulties in making the synthetic transformation. The literature reports only a
select few examples of fluorinated NIR cyanine dyes (Figure 4.2). Fluorinations at the N-alkyl positions and aryl positions both resulted in highly fluorescent/photostable derivatives by comparison to their non-fluorinated counterparts. Even more challenging is the accessibility of \(^{18}\text{F}\) labeled fluorophores due to the relatively short half-life of the radioisotope. Although there are numerous accounts of \([^{18}\text{F}]\) BODIPY fluorophores attainable via isotopic exchange, there are only two known examples of \(^{18}\text{F}\) labeled cyanine dyes to date (Figure 4.3).

**Figure 4.3** \(^{18}\text{F}\) labeled cyanine dyes reported in the literature.

Priem *et al.* developed the fluorinated dye 5 (Figure 4.3) *via* a prelabeled prosthetic group which required multiple steps to synthesize prior to conjugating it to the dye. Ting *et al.* obtained 6 by incorporating a “boron trap” that was conjugated to the dye *via* a bulky linker. Building on the chemistry developed in Chapter 2, introduction of fluorine by direct substitution of the chlorine on IR780 and creating the corresponding hydrocyanine ROS probe could result in probes
with favourable optical properties. Further, translation of this fluorination to $^{18}$F labeling would be a desirable approach to accessing PET agents capable of emitting in the NIR from commercially available meso-chlorine containing dyes, without the need for a prosthetic group.

4.2 Objectives

The goal was to develop the means to synthesize and evaluate fluorinated NIR dyes structurally analogous to that synthesized in Chapter 2. In an effort to improve in vivo clearance, a new class of derivatives were designed to be less hydrophobic with considerably lower partition coefficients. This includes the synthesis of a sulfonated derivative and an analogue with a shorter N-alkylated chain (i.e. methyl versus propyl). Varying the length of N-alkyl chains on cyanine dyes in particular has been shown to dramatically influence the biodistribution.\textsuperscript{12,13,14} Accordingly, the development of a radiolabeled cyanine dye with variable N-alkyl chains is desirable to further optimize the ROS probe described in Chapter 2.

As a complementary strategy, a method for the direct fluorination of IR780 iodide was developed. There are a number of motivations behind being able to create a fluorinated analogue of IR780. Firstly, the exchange of chlorine with fluorine is likely to be accompanied by an increase in lipophility, an attribute that is known to promote uptake of IR780 in preclinical tumour models.\textsuperscript{12} Secondly, the synthetic strategy can also be used to create a new class of $^{18}$F labeled NIR probes potentially in a single step. In addition to the new chemistry, the optical properties of the fluorinated dyes were determined.
4.3 Synthesis, Characterization and Radiolabeling of NIR Turn-on ROS Sensors with variable partition coefficients

Given the results described in Chapter 2, attempts were made to prepare a less hydrophobic ROS probe. To this end, fluorophore 7 was synthesized in a 61% yield using the optimized Suzuki coupling conditions described in Chapter 2 (Scheme 4.2). Commercially available IR783 was used as starting material where the sulfonated derivative 7 was found to have a calculated clog P of ≈ 3.7. Reduction of 7 using methanolic sodium borohydride was attempted but isolation of 8 proved to be difficult. Attempts to scale up the synthesis to facilitate isolation of the product were unsuccessful.

Scheme 4.2 Synthesis of fluorophore 7 via Suzuki coupling with 6-fluoropyridin-3-yl)boronic acid and structure of anticipated ROS probe 8.

Purification of this material by reverse phase chromatography (e.g. C18 Sep-Pak cartridges and HPLC) resulted in impure and/or decomposed material. The $^1$H NMR of 8 was acquired by reducing 7 in MeOH-d4 which revealed the presence of vinyl protons in varying chemical environments, indicative of reduction of the
iminum bond and the absence of symmetry brought about by loss of extended conjugation (Figure 4.4).

**Figure 4.4** $^1$H NMR of 8 derived from borohydride reduction of 7 in MeOH-d4

The excitation and emission spectra were collected in order to determine whether functionalization at this position influenced the NIR emitting properties (Figure 4.5). Functionalization of cyanine dyes specifically at the meso position has been known to be accompanied by changes in the photophysical properties, notably in the appearance of hypsochromic shifts. These studies revealed that 7 experiences an excitation maximum at $\lambda_{\text{ex}}$ 766 nm and an emission maximum of $\lambda_{\text{em}}$ 796 nm. The functionalization resulted in comparable values to the parent compound IR783 ($\lambda_{\text{ex}}$ 766 nm, $\lambda_{\text{em}}$ 798 nm).
Figure 4.5 a) Absorbance spectrum of 7 (40 µM in methanol) b) Corresponding excitation spectrum (λ\text{ex} = 766 nm).

Due to challenges in isolating 8 an alternative strategy was pursued. Previously employed Suzuki coupling conditions were used to synthesize fluorophore 9a and fluorescent intermediate 10 using commercially available intermediate IR775 chloride. This scaffold possesses two less ethyl groups by comparison to IR780 and a calculated clog P value of 11.2. Reduction of the iminium cation using previously established conditions resulted in 11a and radiolabeling precursor 12 (Scheme 4.3). This probe exhibits no overall charge and the cationic intermediate generated from elevated ROS concentrations permits intracellular entrapment of the probe.\textsuperscript{12a}

Excitation and emission scans of a solution of 9a in methanol revealed that the fluorophore exhibited maxima at 762 nm and 788 nm respectively giving a Stokes shift of 26 nm (Figure 4.6). This Stokes shift value is comparable to other cyanine dyes reported in the literature\textsuperscript{16} and is identical to that reported for the previously synthesized ROS probe described in Chapter 2.
Scheme 4.3 Synthesis of ROS probe 11a and radiolabeling precursor 12.

Excitation of the reduced probe 11a at 758 nm showed negligible fluorescence (Figure 4.7). The cyanine dye 9a exhibits a 35-fold increase in fluorescence intensity as compared to 11a at this excitation wavelength. This contrasting difference in intensity is a significant trait for “turn on” probes where background signals can be considerably reduced for in vitro and in vivo studies.¹⁷

Figure 4.6 a) Absorbance spectrum of 9a (40 µM in methanol) b) Corresponding excitation spectrum (λ_{ex} = 762 nm).

The radiolabeling precursor 12 was synthesized by subjecting 10 to methanolic borohydride and stirring for a few seconds at room temperature. The reduction of the iminium bond was kinetically more favourable than the reduction of the nitro
group. Tailoring reaction conditions resulted in preferential reduction of the iminium bond and retention of the nitro group. Altering reaction conditions such as reaction time or borohydride concentrations resulted in complete reduction of the nitro functionality which was detrimental to radiolabeling yields. The nitro functionality was used over the more labile trimethylammonium triflate leaving group as attempts to synthesize this precursor required a greater number of synthetic steps, and resulted in poor yields and challenging purification protocols (data not shown).

![Figure 4.7](image)

**Figure 4.7** Emission spectrum of 40 µM solutions of 9a and 11a in methanol (λ<sub>ex</sub> = 762 nm).

<sup>18</sup>F radiolabeling of 12 was performed in one step, analogously to the ROS probe reported in Chapter 2. The total synthesis/purification time (excluding the drying step) was 35 minutes (RCY = 65% decay-corrected, n = 3). Stability studies performed on 11b (in 10% ethanol in 0.9% saline) revealed that this probe was
more susceptible than the probe described in Chapter 2 to radiolysis brought about by positron-induced ROS formation (Figure 4.8).

![HPLC chromatograms](image)

**Figure 4.8** HPLC chromatograms after formulation of 11b in 10% ethanol/0.9% saline showing stability profile of carrier added mixture. UV trace of 11a (top) and γ-trace (bottom) revealing presence of 9b and the appearance of a new unidentified peak at 31 minutes.

Reinjection shortly after formulation revealed a prominent peak at 31 minutes along with 11b, which was not observed for the previous probe until left in the formulation for 60 minutes. This issue was resolved by the addition of a 0.5 mg/mL solution of ascorbic acid to the formulation which resulted in stabilization of the tracer for up to 4 hours at room temperature (Figure 4.9).

With the stability of 11b resolved, the probe can be evaluated in various *in vivo* preclinical tumour or ROS models. This radiotracer can be contrasted with the previously established probe described in Chapter 2 and compared to the
biodistribution of its oxidized form to determine the \textit{in vivo} stability. These biological studies are essential in determining whether the chain length has an effect on the overall biodistribution.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4_9.png}
\caption{\textgamma-HPLC chromatograms following the incubation of 11b in 10\% ethanol/saline in the presence of ascorbic acid (0.5 mg/mL) a) initial injection b) 2 hours c) 4 hours.}
\end{figure}

4.4 Synthesis, Characterization and Biological evaluation of a Fluorinated Analogue of IR780

4.4.1 Synthesis and Reaction Optimization

As previously discussed, fluorinated dyes are often associated with favourable photochemical properties but accessibility of these probes is limited due to synthetic challenges associated with many fluorination reactions. A nucleophilic fluorination strategy is desirable due to its potential application in $^{18}\text{F}$ PET
chemistry. Cyanine dyes bearing a meso chlorine functionality have been shown to undergo reactions with nucleophiles as a result of the delocalization of electrons on the backbone of the molecule, leading to an electron-deficient π-system.\textsuperscript{18,19} There is considerable debate over the exact mechanism of nucleophilic displacement and it is thought that cyanine dyes undergo these transformations via one of two mechanisms.\textsuperscript{20} The first mechanism is an addition-elimination mechanism facilitated by the cationic charge on the molecule (Figure 4.10a). The second possible mechanism involves a S\textsubscript{NR}1 pathway whereby a single electron transfer (SET) is initiated by the nucleophile (Figure 4.10b, equation 1). This process involved dissociation of R-Cl to the radical cation R\textsuperscript{+} (equation 2) and reaction of this species with the nucleophile (Nu) generates a radical intermediate (equation 3). This intermediate behaves as an electron donor for the propagation process (equation 4).

Displacement of the chloride to synthesize a fluorinated analogue is expected to be particularly challenging due to the poor nucleophilicity of the fluoride anion. Additionally, as expected with typical halogen exchange (HALEX) reactions, harsh conditions such as elevated temperatures and long reactions times are often required.\textsuperscript{21} This poses a challenge for application to cyanine dyes as prolonged heating has been known to promote decomposition products.\textsuperscript{19} Accordingly, fluorination via transition metal catalysis may aid in decreasing reaction times and/or temperatures.
There has been a great interest in the use of transition metal catalysis (such as copper\textsuperscript{22} and palladium\textsuperscript{23}) for the conversion of aryl/vinyl (pseudo) halides to their corresponding fluorinated products. Initially, the fluorination of IR780 was attempted under standard conditions in the absence of transition metal catalysts using DMSO as the solvent and KF as the fluoride source (Table 4.1, entries 1,2, 4,5). These reactions resulted in poor yields, recovery of starting material or the formation of a byproduct.
Table 4.1 HALEX conditions for the fluorination of IR780. BP = by-product; NR = no reaction.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Temperature (°C)</th>
<th>Base</th>
<th>Solvent</th>
<th>Catalyst/additive</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>150</td>
<td>K$_2$CO$_3$</td>
<td>DMSO</td>
<td>18-crown-6</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
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<td>150</td>
<td>K$_2$CO$_3$</td>
<td>DMSO</td>
<td>K$_{222}$</td>
<td>2%</td>
</tr>
<tr>
<td>5</td>
<td>150</td>
<td>K$_3$PO$_4$</td>
<td>DMSO</td>
<td>18-crown-6, Cu$_2$(OTf)$_2$</td>
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<td>6</td>
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<td>-</td>
<td>DMSO</td>
<td>18-crown-6, Cu$_2$(OTf)$_2$</td>
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<tr>
<td>7</td>
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<tr>
<td>8</td>
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<td>CsF</td>
<td>CH$_3$CN</td>
<td>18-crown-6, Cul</td>
<td>NR</td>
</tr>
<tr>
<td>9</td>
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<td>CsF</td>
<td>t-BuOH</td>
<td>-</td>
<td>NR</td>
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<tr>
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<td>t-BuOH</td>
<td>-</td>
<td>NR</td>
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<tr>
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<tr>
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<td>K$_3$PO$_4$</td>
<td>DMSO</td>
<td>Cul</td>
<td>30%</td>
</tr>
<tr>
<td>14</td>
<td>150</td>
<td>Cs$_2$CO$_3$</td>
<td>DMSO</td>
<td>Cul</td>
<td>BP/NR</td>
</tr>
<tr>
<td>15</td>
<td>150</td>
<td>K$_3$PO$_4$</td>
<td>DMSO</td>
<td><strong>14a</strong>, Cul</td>
<td>65%</td>
</tr>
<tr>
<td>16</td>
<td>150</td>
<td>K$_3$PO$_4$</td>
<td>DMSO</td>
<td><strong>14b</strong>, Cul</td>
<td>32%</td>
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<tr>
<td>17</td>
<td>150</td>
<td>K$_3$PO$_4$</td>
<td>DMSO</td>
<td><strong>14a</strong>, Cu(OTf)$_2$</td>
<td>68%</td>
</tr>
<tr>
<td>18</td>
<td>150</td>
<td>K$_3$PO$_4$</td>
<td>DMSO</td>
<td>Cu(OTf)$_2$</td>
<td>16%</td>
</tr>
<tr>
<td>19</td>
<td>150</td>
<td>K$_3$PO$_4$</td>
<td>DMSO</td>
<td><strong>16</strong>, Cul</td>
<td>48%</td>
</tr>
<tr>
<td>20</td>
<td>150</td>
<td>K$_3$PO$_4$</td>
<td>DMSO</td>
<td><strong>15</strong>, Cul</td>
<td>BP</td>
</tr>
<tr>
<td>21</td>
<td>150</td>
<td>K$_3$PO$_4$</td>
<td>DMSO</td>
<td><strong>15</strong>, Cu(OTf)$_2$</td>
<td>BP</td>
</tr>
<tr>
<td>22</td>
<td>150</td>
<td>K$_3$PO$_4$</td>
<td>DMSO</td>
<td>Pd(OAc)$_2$, t-BuXPhos</td>
<td>BP</td>
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Interestingly, the use of phase transfer catalysts in the absence of additional base and in the presence of copper (I) resulted in higher yields (entry 3).

Similarly, the addition of stoichiometric additives commonly employed in nucleophilic fluorination reactions such as t-BuOH\textsuperscript{24} (entry 10) and TBAF\textsuperscript{25} (not shown) resulted in no conversions. The latter may be due to catalyst poisoning brought about by the iodide counterion.\textsuperscript{25} The reaction required a minimum of $140^\circ$C for it proceed which is typical of HALEX reactions.\textsuperscript{26} The addition of base increased the yields, where K$_3$PO$_4$ afforded greater yields than K$_2$CO$_3$ (entry 4 and 5). This finding is in agreement with a previous report.\textsuperscript{23b} Interestingly, employing Cs$_2$CO$_3$ resulted in either no reaction or the generation of a byproduct (entry 13 versus entry 14). The choice of the base has been reported to be important in stabilizing the catalyst complex.\textsuperscript{23b}

CsF as both the source of fluoride and the base showed no conversion to the product (entries 8 and 9). As an alternative KF was used as the fluoride source because it is inexpensive and less hygroscopic than other fluoride sources, which adds to the practicality of the reaction.\textsuperscript{23b} Unlike the fluorination of alkyl triflates reported by Lalic, the use of dried KF was not necessary for this reaction to occur.\textsuperscript{22d} However, due to the hygroscopic nature of the solvent (DMSO) it is thought that the identity of the byproduct may be 17. A peak in the MS spectrum of the crude reaction mixture (521) corresponds to the molecular weight of structure 17.
The use of palladium catalysts in combination with bulky, phosphine ligands has been explored for fluorination of aryl/vinyl (pseudo) halides. Buchwald has shown through mechanistic studies that the use of the bulky phosphine monodendate ligands facilitates C-F reductive elimination reactions which are known to be the rate determining step in these type of cross-coupling transformations. Furthermore, the use of such ligands suppresses the favourable formation of the P-F bond. For this reason, fluorination was attempted using Pd catalysts in combination with monodendate phosphine ligands (for example, entry 22). This resulted in the formation of what is believed to be 17. The results suggest that with this catalyst system hydroxylation is preferential over the desired fluorination (see below for further details).

Conversions to 13 were more successful with copper catalysis. No significant difference in yields were observed in employing Cu (I) versus Cu (II) catalysts (entries 15 and 17). This is contrary to findings reported by Lu et al., where they noted a remarkable difference in Cu (I) and Cu (II) catalysts in hydroxyl-trifluoromethylation reactions. The use of copper catalysts in combination with N-heterocyclic carbenes (NHC) has been successful in high yielding fluorination of alkyl triflates using KF as the fluoride source under mild conditions. We used NHC source 16 in combination with CuI where the reaction afforded 13 in a 48% yield (entry 19). Reducing temperatures resulted in no conversions (data not shown). Interestingly, the use of the diamine ligand 15, known to form strong and stable Cu complexes, resulted in poor yields of the fluorinated product (entry 20)
This may suggest that this reaction does not proceed in a typical cross-coupling type mechanism, where the stabilization of a Cu (III) complex is necessary for the catalytic cycle. Dang et al. have suggested that the use of a copper complex may promote a phase transfer catalysis in the organic solvent and thus improve the nucleophilicity of the fluoride. In their study, the use of TEMPO as a radical scavenger ruled out the possibility of a radical mechanism reaction.

Although phosphine ligands are more commonly employed with Pd catalysts, previous employment of Cu-monodendate phosphine complexes (including those derived from PA) have been successful in performing various transformations including 1,4 hydroxytrifluoro methylations. This reaction like the one described here is also dependent on the base used (entry 12 and entry 15).

It is important to note that the use of a ligand is not absolutely necessary to achieve the desired transformation (entry 13), but does improve yields. Mechanistic studies on allylic/vinyllic fluorinations suggest that the metal binds to the alkyl ligand generating an intermediate much like that observed in the Heck-Mizoroki reaction. Copper is known to form stable complexes with alkenes. Therefore, it is plausible that one or more of the vinyl groups present on IR780 may participate in copper complexation, facilitating the formation of the C-F bond. The proposed mechanism involves Cu ligation to the alkene to form a Cu (III) intermediate where halide exchange can take place. This is then followed by nucleophilic attack and subsequent β-elimination (Figure 4.11). This mechanism relies on the ability of the metal to ligate to the alkene which would significantly weaken the alkyl bond.
and facilitate the reaction by decreasing the demanding energy barrier. This may further explain why reactions with strongly binding ligand 15 preferred the formation of the byproduct (17) which could have prevented metal-ligation to the alkene.

\[ \text{Cl} - \text{R} \xrightarrow{\text{CuX}} \text{Cl} - \text{F} - \text{Cu-L}_n \xrightarrow{\text{elimination}} \text{F} - \text{R} \]

18 19

**Figure 4.11** Proposed mechanism for copper catalyzed fluorination of IR780 via Cu (III) intermediate 19.

The optimal reaction conditions required stoichiometric amounts of copper catalyst, equal equivalents of base and 0.5 eq of the PA ligand (see Appendix 2 for supporting characterization of this compound). Potassium fluoride was used in 10-fold excess and the reaction was complete within 30 minutes of heating at 150°C. Decreasing temperatures below 140°C resulted in no reaction. Reactions left overnight at 80°C also resulted in the formation of 17. Preliminary investigations on substrate scope demonstrate that this reaction can be translated to other cyanine dyes, such as IR783 and IR775 as determined by HRMS. However, isolation of the desired products are complicated by the presence of byproducts or starting materials which often coelute with the product and thus complete characterization of these particular dyes is still required.
4.4.2 Optical properties and Biological evaluation

The optical properties of 13 were assessed at a concentration of 40 µM and compared to IR780 iodide under similar conditions. The absorption and emission maxima observed for 13 exhibited a hypsochromic shift by 20 and 26 nm respectively. Such hypsochromic shifts have been observed for previously synthesized fluorinated dyes.\textsuperscript{8,32} Absorbance shifts to lower wavelengths often result in the formation of photostable probes and ones that are less prone to photodegradation.\textsuperscript{32} The emission profile of 13 also demonstrated a 4-fold increase in fluorescence intensity as compared to IR780 (Figure 4.12).

ROS probe hydro-IR780 has been shown to increase sensitivity of commercial HRP ELISA assays by two orders of magnitude.\textsuperscript{33} To further assess whether the sensitivity of 13 was translatable in vitro, IR780 and 13 were reduced to synthesize the corresponding hydrocyanines which were then evaluated in a PC-3 cell assay (Scheme 4.4).

![Figure 4.12 Absorbance and emission profiles of IR780 iodide (\(\lambda_{\text{ex}} = 776\) nm, \(\lambda_{\text{em}} = 808\) nm) and 11 (\(\lambda_{\text{ex}} = 756\) nm, \(\lambda_{\text{em}} = 782\) nm) at a concentration of 40 µM in acetonitrile a) absorbance spectrum b) emission spectrum.](image-url)
Compound 20 and hydro-IR780 were incubated with PC-3 cells at concentrations of 100, 150 and 200 μM for 120 minutes after which the fluorescence was measured (Figure 4.13). At each concentration, 20 showed a 2-2.5 fold increase in fluorescence intensity when incubated with PC-3 cells as compared to hydro-IR780 ($p < 0.001$).

**Figure 4.13** PC-3 cell assay study: fluorescence response of 20 and hydro-IR780 after a 120 minute incubation period with PC-3 cells ($p < 0.001$).
Turn-on ratios (fluorophore incubated with PC-3 cells versus untreated fluorophore) for 18 were between 3.6 and 4-fold where by comparison, hydro-IR780 exhibited a 1.4-2.3-fold increase at all concentrations tested. The enhanced fluorescence response implies that lower concentrations of the probe 18 can be used to assess ROS activity in vitro and in vivo.

4.4.3 Attempted radiolabeling of IR780 iodide

Copper-assisted $^{18}$F labelings have been successfully performed on aryl iodides$^{34}$ (via formation of $^{18}$F trifluoromethylated products), aryl iodonium salts$^{35}$ and aryl boronic esters.$^{36}$ These reactions, as the one described, are highly dependent on the substrate used. We attempted the labeling using 5 mg (0.007 mmol) of IR780 iodide. After azeotropic drying, 1mg of CuI was added to the vial followed by the addition of the dye in dry DMSO and the reaction was heated to 160°C for 4 minutes.

![Figure 4.14](image_url)

**Figure 4.14** Crude HPLC chromatograms: UV trace (bottom) and γ-trace (top) revealing peak that corresponds to the retention time of 13.
The crude chromatogram showed the appearance of a radioactive peak at 22 minutes corresponding to the retention time of IR780 iodide and the desired product 13. The reaction however, was very difficult to reproduce, after multiple attempts and variable reaction conditions.

4.5 Summary and Conclusions

This chapter describes the synthesis of fluorine containing cyanine dyes. The main aim was to develop a synthetic platform that would allow access to fluorescent probes, with the ultimate goal of developing $^{18}\text{F}$ labeled multi-modal probes. The synthesis of structural analogues of the probe described in Chapter 2 was established, including one example which possessed a shorter N-alkyl chain. This analogue was successfully radiolabeled and stability studies demonstrated this probe was stable in solution at 4 hours in the presence of a formulation containing ascorbic acid. These probes also retained emission wavelengths in the NIR. Further, a fluorinated analogue of IR780 was successfully developed via an optimized transition-metal catalyzed HALEX reaction. Although the radiolabeling of this probe was not reproducible, the corresponding reduced ROS probe showed enhanced fluorescence both in solution and in a PC-3 cell assay in comparison to hydro-IR780. The sensitivity of this probe is a favourable trait and can be advantageous during in vitro and in vivo studies.
4.6 Experimental

Supplementary figures can be found in APPENDIX 2

Synthesis of 7

IR783 (29 mg, 0.04 mmol), 6-fluoropyridin-3-ylboronic acid (7.0 mg, 0.05 mmol), Pd(OAc)$_2$ (1.0 mg, 10 mol%) and t-BuXPhos (3.0 mg, 20 mol%), were added to a vial equipped with a Teflon® cap and purged under argon for 2 minutes. The contents of the vial were suspended in ethanol (1 mL) and argon bubbled through the solution for 5 min using a long needle. K$_3$PO$_4$ (aq) (0.5 mL, 0.2 mM) was added under argon and the vial sealed, covered with aluminum foil and heated at 100°C overnight. The reaction vessel was cooled and the reaction mixture diluted with water (2 mL) and directly loaded onto a RediSep® Rf 18C reverse phase column (using 30% water in acetonitrile as the eluent) to afford 5 as a green solid (19 mg, 61%). $^1$H NMR (700 MHz, MeOH-d$_4$) δ 8.12 (d, $J = 2.2$ Hz, 1H), 7.90 (td, $J_1 = 7.9$ Hz, $J_2 = 2.3$ Hz, 1H), 7.38 (m, 5H), 7.30 (d, $J = 7.8$ Hz, 2H), 7.19 (t, $J = 7.4$ Hz, 2H), 7.14 (d, $J = 13.9$ Hz, 2H), 6.29 (d, $J = 13.9$ Hz, 2H), 4.15 (t, $J = 7.1$ Hz, 4H), 2.87 (t, $J = 7.3$ Hz, 4H), 2.78 (t, $J = 6.1$ Hz, 4H), 2.07 (m, 2H), 1.96 (m, 4H), 1.91 (m, 4H), 1.26 (s, 12H). $^{13}$C NMR (175 MHz, MeOH-d$_4$) δ 173.5, 164.9 ($J = 241.1$ Hz), 157.2, 149.1 ($J = 14.1$ Hz), 148.4, 145.0 ($J = 7.8$ Hz), 142.8 ($J = 250.1$ Hz), 134.6 ($J = 4.2$ Hz), 133.7, 129.9, 126.2, 123.4, 112.1, 110.1 ($J = 37.3$ Hz), 101.2, 51.8, 50.0, 44.8, 28.0, 27.9, 27.2, 25.8, 23.6, 22.4 HRMS (ESI+) for C$_{43}$H$_{49}$FN$_3$O$_6$S$_2$ calcd. 786.3047 Obsd: 786.3030
Synthesis of 9a

IR775 chloride (20 mg, 0.04 mmol), 6-fluoropyridin-3-ylboronic acid (7.0 mg, 0.05 mmol), Pd(OAc)$_2$ (1.0 mg, 10 mol%) and t-BuXPhos (3.0 mg, 20 mol%), were added to a vial equipped with a Teflon® cap and purged under argon for 2 minutes. The contents of the vial were suspended in ethanol (1 mL) and argon bubbled through the solution for 5 min using a long needle. K$_3$PO$_4$ (aq) (0.5 mL, 0.2 mM) was added under argon and the vial sealed, covered with aluminum foil and heated at 100°C overnight. The reaction vessel was cooled and the reaction mixture diluted with water (10 mL) and extracted with DCM (2 × 10 mL). The organic phases were combined, dried over anhydrous MgSO$_4$ and concentrated in vacuo.

The desired product was isolated using a 8 gram flash RediSep® Rf basic alumina column (using 10% MeOH in DCM as the eluent) to afford 2 as a green solid (17 mg, 73%) $^1$H NMR (600 MHz, MeOH-d$_4$) δ 8.11 (s, 1H), 7.89 (td, $J_1 = 7.9$ Hz, $J_2 = 2.5$ Hz), 7.39 (m, 5H), 7.26 (d, $J = 7.9$ Hz, 2H), 7.21 (td, $J_1 = 7.5$ Hz, $J_2 = 0.7$ Hz, 2H), 7.15 (d, $J = 14.0$ Hz, 2H), 6.23 (d, $J = 14.0$ Hz, 2H), 3.60 (s, 6H), 2.77 (t, $J = 6.2$ Hz, 4H), 2.07 (m, 2H), 2.01 (s, 1H), 1.72 (d, $J = 8.1$ Hz, 1H), 1.27 (s, 12 H). $^{13}$C NMR (150 MHz, MeOH-d$_4$) δ 174.2, 164.9 ($J = 241.2$ Hz), 157.1, 149.1 ($J = 14.1$ Hz), 148.3, 144.9 ($J = 7.8$ Hz), 143.2 ($J = 329.9$ Hz), 134.5 ($J = 3.6$ Hz), 133.3, 129.8, 126.2, 123.3, 111.8, 110.1 ($J = 37.2$ Hz), 101.6, 31.6, 27.9, 27.7 25.8, 22.3. HRMS (ESI+) for C$_{37}$H$_{39}$FN$_3$ calcd. 544.3128 Obsd: 544.3141.

Synthesis of 10
IR775 chloride (20 mg, 0.04 mmol), 2-nitro-5-pyridineboronic acid pinacol ester (12 mg, 0.05 mmol), Pd(OAc)$_2$ (1.0 mg, 10 mol %) and $t$-BuXPhos (3.0 mg, 20 mol %) were added to a vial equipped with a Teflon® cap and purged under argon for 2 min. The contents of the vial were suspended in absolute ethanol (1 mL) and argon bubbled through the solution for 5 min using a long needle. K$_3$PO$_4$ (aq) (0.5 mL, 0.2 mM) was added under argon and the vial sealed, covered with aluminum foil and heated at 100°C overnight. The reaction vessel was cooled and the reaction mixture diluted with water (10 mL) and extracted with DCM (2 × 10 mL). The organic phases were combined, dried over anhydrous MgSO$_4$ and concentrated in vacuo. The product was isolated using a 8 gram flash RediSep® Rf basic alumina column (using 20% MeOH in DCM as the eluent) to afford 4 as a green solid (14 mg, 57%). $^1$H NMR (600 MHz, MeOH-d$_4$) δ 8.61 (d, $J = 8.1$ Hz, 1H), 8.57 (d, $J = 2.1$ Hz, 1H), 8.20 (dd, $J = 8.1$ Hz, 1H), 7.37 (d, $J = 7.4$ Hz, 4H), 7.26 (d, $J = 7.8$ Hz, 2H), 7.21 (m, 2H), 6.99 (d, $J = 14.0$ Hz, 2H), 6.26 (d, $J = 14.0$ Hz, 2H), 3.61 (s, 6H), 2.79 (t, $J = 6.21$ Hz, 4H), 2.10 (m, 2H), 1.22 (d, $J = 5.9$ Hz, 12H). $^{13}$C NMR (150 MHz, MeOH-d$_4$) δ 174.3, 157.9, 155.3, 150.6, 147.8, 144.2, 143.5, 143.1, 142.1, 133.2, 129.8, 126.4, 123.3, 119.2, 111.9, 101.9, 31.6, 27.8, 27.7, 25.8, 22.3. HRMS (ESI+) for C$_{37}$H$_{39}$N$_4$O$_2$ calcd. 571.3073 Obsd: 571.3080.

Reduction of 9a was performed in accordance with the method used for the ROS probe described in chapter 2. Briefly, compound 9a (9 mg, 0.016 mmol) was dissolved in MeOH (2 mL) in a 30 mL scintillation vial covered with aluminum foil. Freshly prepared NaBH$_4$ in MeOH (1 mL, 0.16 mM) was added dropwise to the
green solution, generating a yellow solution. The reaction was stirred at room temperature (RT) for 10 min and the solvent was removed by rotary evaporation. The resulting solid was dissolved in DCM (3 mL) and extracted with water (2 × 3 mL). The organic phase was dried over anhydrous MgSO₄ and the solvent was removed by rotary evaporation to afford a bright yellow solid.

**Spectroscopic studies**

Fluorescence measurements were obtained using a Tecan® infinity M1000 plate reader. Briefly, the dyes were suspended in MeOH or CH₃CN to make a stock solution of known concentration which was used to make up a 40 μM solution. 150 μL of the solution were deposited into a 96-well plate which were used for excitation/emission readings. These values were subtracted from an equal volume of the blank (pure solvent).

**Radiolabeling Procedure**

Compound 10 (4.2 mg, 0.007 mmol) was dissolved in 400 μL of MeOH, and 200 μL of a freshly prepared 0.03 mM sodium borohydride solution in MeOH was added slowly. An instant colour change from green to yellow was observed. The reaction was stirred at RT for 30 s after which it was extracted with DCM (2 × 10 mL). The organic phase was dried over anhydrous MgSO₄ and the solvent was removed by rotary evaporation to afford the reduced product as a yellow-brown solid (3.1 mg). A solution of K[^¹⁸F] F-K₂₂₂ complex (0.92 GBq) was transferred to a 5 mL pyrex® reaction vial and dried at 85°C in an oil bath under a stream of nitrogen. Azeotropic
drying by the addition of 1 mL portions of CH$_3$CN was performed over a 20-minute period (total of 6 mL). The reduced precursor was dissolved in 400 μL of dry DMF and added to the $^{18}$F/K$_{222}$ mixture which was subsequently heated at 150°C for 4 minutes. After cooling to RT the desired product was isolated by semi-preparative HPLC (decay corrected RCY = 62% ± 5, n = 3).

**Stability profile of 11b**

Compound 11b (19.98 MBq) was suspended in 600 μL of 10% ethanol/saline and incubated at RT. The solution was analyzed immediately after formulation by reverse-phase HPLC (95%water/CH$_3$CN gradient).

Stability was also assessed in the presence of ascorbic acid. Compound 11b (21.98 MBq) was suspended in 500 μL of 10% ethanol/saline. To this was added 100 μL of a 0.5 mg/mL aqueous solution of ascorbic acid. The mixture was left to incubate at RT and the solution was analyzed by reverse-phase HPLC at 2 and 4 hours.

**Synthesis of 13**

IR780 (25 mg, 0.04 mmol), KF (23 mg, 0.4 mmol), Cu(OTf)$_2$ (14 mg, 0.04 mmol), 12a (2 mg, 0.0007 mmol) and K$_3$PO$_4$ (8.5 mg, 0.04 mmol) were added to a vial equipped with a Teflon® cap and purged under argon for 2 minutes. The contents of the vial were suspended in DMSO (600 μL) and the vial was sealed and heated to 150°C for 30 minutes. The reaction vessel was cooled and the mixture was diluted with water (10 mL) and extracted with DCM (2 x 10 mL). The organic phases were combined, dried over anhydrous MgSO$_4$ and concentrated *in vacuo.*
The desired product was isolated using a 8 gram flash RediSep® Rf basic alumina column (using 3% MeOH in DCM as the eluent) to afford 11 as a blue-green solid (17 mg, 68%). $^1$H NMR (600 MHz, CD$_2$Cl$_2$) δ 8.05 (d, $J = 14.2$ Hz, 2H), 7.37 (m, 4H), 7.24 (t, $J = 7.4$ Hz, 2H), 7.11 (d, $J = 8.0$ Hz, 2H), 6.05 (d, $J = 14.2$ Hz, 2H), 4.07 (t, $J = 7.2$ Hz, 4H), 1.97 (m, 2H), 1.89 (q, $J_1 = 7.4$ Hz, $J_2 = 7.4$ Hz, 4H), 1.67 (s, 12H), 1.24 (m, 2H), 1.07 (t, $J = 7.4$ Hz, 6H) $^{13}$C NMR (150 MHz, MeOH-d4) δ 172.2, 142.5, 141.0, 140.1 ($J = 12.3$ Hz), 128.9, 125.3, 122.3, 116.9, 110.9, 100.0, 49.4, 46.3, 41.2, 28.3, 24.6, 21.0, 11.9 HRMS (ESI+) for C$_{36}$H$_{44}$N$_2$F calcd. 523.3489 Obsd: 523.3503.

**PC-3 cell assay**

The PC-3 cell line purchased from ATCC (CRL-1435). Cells were propagated using Ham's F-12 Nutrient Mix supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin Streptomycin at 37°C and 5% CO$_2$ and were not used beyond passage 30. Hydro IR780 and 20 were prepared in DMSO to a concentration of 5 mM immediately before use. PC-3 cells (8.5 × 10$^4$ cells/well) were loaded on a 96 well plate and allowed to attach overnight. Growth media was removed and cells were washed twice with 0.2mL of PBS. 0.2mL of 0 µM, 100 µM, 150 µM and 200 µM of TA in 20.5mM HEPES pH 7.5 was loaded onto the cells including no cell and vehicle only controls. The plate was incubated for 2h at 37°C and 5% CO$_2$. Fluorescence was measured ($\lambda_{ex}$ / $\lambda_{em}$ = 756 nm/782 nm for 20, $\lambda_{ex}$ / $\lambda_{em}$ = 768 nm/794 nm for hydro-IR780) using a Tecan Infinite M1000. Cells were lysed with 0.05mL of 1% Triton X-100 and incubated at 37°C for 30min with gentle shaking.
A sample from each well was collected and the amount of protein was determined using the Pierce BCA Protein Assay Kit in order to normalize each sample by µg of protein.

4.7 References


Chapter 5: Towards the Synthesis and Evaluation of TACE Inhibitors as Potential Molecular Imaging Probes for Rheumatoid Arthritis

5.1 Introduction

TNF-α converting enzyme (TACE or ADAM 17) belongs to a class of enzymes known as ADAM enzymes (A disintegrin and metalloproteinase), which are a close relative of another prominent class of peptidases known as matrix metalloproteases (MMPs). TNF-α converting enzyme is a transmembrane glycoprotein that behaves as a sheddase enzyme and exists as a metalloprotease where zinc is tightly bound by free cysteine residues.\(^1\) The importance of metalloproteases in human physiology and their active involvement in biochemical processes is emphasized by the fact that metalloproteases make up 33% of the proteases encoded by the human genome.\(^2\) Inhibiting over-active TACE can disable proinflammatory effects of TNF-α by inhibiting the process by which pro-TNF-α is converted to s-TNF-α. TACE catalyzes this conversion by hydrolyzing the Ala 76 and Val 77 bond, shedding the 17 KDa protein responsible for the proinflammatory cascade of events.\(^3\) TACE has become a very sought after clinical target, as it is responsible for the processing of 90% of TNF-α.\(^4\) The enzyme is also actively involved in processing various ligands such as EGF and β-APP which are directly involved in the development of cancer and Alzheimer’s disease respectively, making it an especially significant therapeutic target (Figure 5.1).\(^5\)

The over-expression of the cytokine TNF-α is seen as the chief initiator of the proinflammatory response and has also been linked to many pathological
conditions including Crohn’s disease\textsuperscript{6}, ulcerative colitis\textsuperscript{6}, cancer\textsuperscript{7} and Alzheimer’s disease.\textsuperscript{8} The clinical significance of TNF-α blockage has been shown to dramatically improve patient prognosis in several disease states.\textsuperscript{3} One notable example demonstrated that TACE inhibition reverts the breast cancer malignant phenotype in the T4-2 cell line to morphology characteristic of non-malignant cells.\textsuperscript{9}

**Figure 5.1** Schematic showing the various ligands processed by TACE/ADAM17 and its involvement with multiple disease states.\textsuperscript{5}

Currently, TNF-α inhibition has been accomplished by the use of therapeutic biologics such as adalimumab (Humira®). Small-molecule inhibition of TACE resulted in failed clinical trials due to severe musculoskeletal side effects.\textsuperscript{10} This is thought to be attributed to the non-selective nature of these drugs which are
capable of also inhibiting MMP-1 and/or MMP 14.\textsuperscript{11} The inability to achieve selective targeting of TACE is one of the reasons for the lack of availability of these drugs.

5.2 Objectives

In nuclear imaging, toxicity is less often observed due to the low mass dose of the tracer used (nmol to pmol). Molecular imaging of the inflammatory process can provide an early detection and therapeutic monitoring tool that can be used in place of conventional anatomical imaging modalities which can prevent late-stage irreversible bone damage.\textsuperscript{12} Recent studies have shown the feasibility of molecularly imaging RA using \textsuperscript{99m}Tc labeled anti-TNF-\alpha antibodies infliximab\textsuperscript{12,13} and adalimumab.\textsuperscript{13} The scintigraphic images obtained in these studies have demonstrated the importance of early detection and therapeutic intervention where reversing the molecular events responsible for pathological progression may be feasible. While radiolabeled monoclonal antibodies demonstrate rapid uptake exclusively in diseased tissue, this particular strategy is expensive and generally exhibits poor pharmacokinetics as a result of the relatively large size of the probe.

Radiolabeled analogues of TACE inhibitors may be an effective means for the molecular imaging of RA. TACE has never been used for such a purpose but it is localized and over expressed in the synovium of RA patients.\textsuperscript{4} TACE inhibitors\textsuperscript{14,15} that were selected as platforms to create new tracers were reported as being highly potent and selective ligands (Figure 5.2). These inhibitors possess hydroxamates/hydroxamic acids, which provide potency by binding zinc in the
active site and a quinolone that imparts selectivity towards TACE.¹⁶ Inhibitor 1 is a sulfone-based TACE inhibitor that possesses a prominent reverse hydroxamate functionality, which was reported to be less prone to hydrolysis in vivo. TACE inhibitor 2 was pursued as a result of its metabolic stability, its affinity in whole blood assays and fast clearance rate.

\[
\text{IC}_{50} = 2.8 \text{nM} \\
2a: X = O, K_i = 0.35 \text{nM}^* \\
2b: X = -\text{NCH}_2\text{CH}_2\text{F}, \text{IC}_{50} = 2.6 \text{nM}^*
\]

* in vitro assay performed on pTACE

Figure 5.2 Structures of two selective and high affinity TACE inhibitors being pursued as targeting vectors for probe development.

The goal was to synthesize non-radioactive derivatives of these compounds and to test the analogues in an established TACE assay to ensure structural modifications retain TACE activity. Following successful validation, radiolabeling precursors would be synthesized in order to produce the radiotracer for in vitro and in vivo screening.
5.3 Synthesis and in vitro validation of TACE inhibitors

Work began by synthesizing inhibitors 1, 2a and 2b to validate the in vitro testing of the synthesized compounds as well as to provide tractable synthetic methods prior to structural modification of the scaffold. The syntheses of some intermediates were modified from the original reported procedures. The synthesis of 1 began with the construction of the P1' substituent fragment of the inhibitor (Scheme 5.1).

Scheme 5.1 Synthesis of P1' fragment of TACE inhibitor.

Quinalidine 3 underwent alpha-hydroxyalkylation\textsuperscript{17} in the presence of ammonium per oxydisulphate to yield 33% of alcohol 4. Treatment of 4 with thionyl chloride afforded 5 which was coupled to sulfone 8 in the presence of cesium carbonate as the base. Sulfone 8 was synthesized in a 92% yield via a nucleophilic copper-assisted displacement\textsuperscript{18} reaction with commercially available 7. Coupling of 5 and 8 in the presence of cesium carbonate afforded 9 in a 64% yield.
Scheme 5.2 displays conditions for the completed synthesis of 1. Incorporation of the 3-methyl pyridine functionality was accomplished through a Julia coupling\textsuperscript{19} with ester 11 derived from commercially available nicotinic acid 10. The ester was used as the electrophile due to failed attempts or low yields associated with the synthesis using the corresponding aldehyde. Ketone 12 was then reduced using sodium borohydride in methanol and the corresponding alcohol 13 was subsequently dehydrated to give 14 in a 78% yield.

The synthesis of 1 was then completed in a two-step manner, where 14 was used to produce the hydroxylamine, followed by prompt formylation. Initially, a “mixed-anhydride” method of formylation was attempted but it was found that both the nitrogen and oxygen of the hydroxylamine were formylated. Various attempts at selective formylation failed, including the use of 2,2,2-trifluoroethylformate (TFEF) as the formylating agent.\textsuperscript{20} Other routes to 1 were pursued including the synthesis
of the oxime via ketone 12, which could be used to directly synthesize the hydroxylamine, but these were unsuccessful (not shown). Similarly, reacting 13 with O,N-Boc protected hydroxylamine in a Mitsunobu reaction failed to afford the corresponding protected hydroxylamine\(^{21}\) (not shown).

Scaffold 2 was synthesized using the conditions shown in scheme 5.3. Quinoline intermediates 17a/17b were prepared in a similar manner to that of the previously described 9. The pyran/piperidine functionality was introduced by initially performing a Horner-Wadsworth-Emmons olefination\(^{22}\) with commercially available reagents 19a/19b to afford 20a and 20b in a 78 and 57% yield respectively. Conjugate addition of 20a/20b led to the transesterified amines 21a/21b which were coupled to 18a/18b through a PYBOP promoted peptide coupling. Originally, the goal was to synthesize 2 directly from esters 22 where standard conditions\(^{23, 24}\) called for the presence of a strong base and that the reaction be performed at ambient temperature as reported in the original communication.\(^{15}\) Performing the reaction under these conditions resulted in only recovery of the starting material and/or the corresponding carboxylic acids (23a-23d), with no evidence of the anticipated hydroxamic acids. This led to a microwave assisted approach where the reaction was performed in either basic methanol\(^{25}\) or aqueous solution.
Scheme 5.3 Synthesis of TACE inhibitors 2a, 2c and derivative 2b.

Both conditions yielded very little of the hydroxamic acid with the majority of the reaction mixture consisting of the starting ester. Consequently, we performed the conversion in two steps, with the initial hydrolysis of the esters followed by a peptide coupling with commercially available O-protected hydroxylamines, which in some cases, were cleaved in situ and afforded 2 in a 48% yield. O-Protected tert-butyldimethyl silyl hydroxylamines and O-tetrahydropyranyl hydroxylamines provided highest product yields, with the former protecting group cleaving in situ.

2b and 2c were synthesized as non-radioactive standards with the intention of creating SPECT and PET radiotracers respectively. Compound 2c was previously assessed in an in vitro assay using porcine TACE and was found to have an IC$_{50}$
of 2.5 nM.\textsuperscript{15} For comparison, TACE activity of 2a, 2b and 2c were tested in an established fluorogenic assay against human recombinant TACE.\textsuperscript{26} Marimastat, a broad-spectrum inhibitor was used as a positive control for the assay (Figure 5.3).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{ic50_curves.png}
\caption{IC\textsubscript{50} curves for 2a, 2b, 2c and positive control marimastat using an established fluorogenic assay.\textsuperscript{26} All experiments were performed in triplicate.}
\end{figure}

While 2a and 2c exhibited low nanomolar inhibitory activity, 2b demonstrated that introduction of iodine at the P1' substituent resulted in a dramatic loss of potency. This led us to pursue the fluorine-18 analogue of 2c. Initially, we envisioned a direct \textsuperscript{18}F labeling via the synthesis of alcohol 24. Tosylation was performed on the protected hydroxamic acid to avoid decomposition via the Lossen rearrangement.\textsuperscript{27} Unsuccessful attempts at mesylating and tosylating under
various reaction conditions led to pursuing an indirect labeling approach strategy (Scheme 5.5). Boc-protected 23c was coupled to O-tertbutyl protected hydroxylamine to afford 27. The Boc protecting group was selectively cleaved in TFA/dichloromethane to afford precursor 28 in a 46% yield (Scheme 5.5).

**Scheme 5.4** Attempted mesylation/tosylation of 24 in an effort to produce labeling precursor 26.

**Scheme 5.5** Synthesis of intermediate 28 and proposed method for subsequent \(^{18}\text{F} \) labeling via alkylation.

Given the success of the work on the ROS probes described in previous chapters, and the challenges in the synthesis of the TACE probes the project was
halted at this stage. Going forward the ditosylate 29 can be labeled to afford 30 which can subsequently be used to alkylate 28 and following deprotection, would afford the desired radiotracer. Previous reports have shown successful $^{18}$F labeling of hydroxamic acid containing MMP inhibitors via nucleophilic substitution, having been performed on both O-tBu protected and naked hydroxamic acids. Due to the thermal demand of the alkylation reaction, intermediate 28 is necessary in order to prevent O-alkylation of the hydroxamic acid.

5.4 Summary and Conclusions

The synthesis of model TACE ligands was achieved however labeling the products was not pursued further. Modified synthesis of two known TACE inhibitors both of which exhibited low nanomolar affinity towards human recombinant TACE was achieved. Radiolabeling precursors were synthesized and can be used to synthesize the desired tracers. The related iodinated compound that was synthesized was not pursued further due to its micromolar affinity.

5.5 Experimental

Synthesis of (2-methylquinolin-4-yl) methanol (4) $^{29}$

To a solution of 2-methylquinoline (1.50 g, 10.4 mmol) and ammonium peroxodisulfate (4.76 g, 20.8 mmol) in methanol/water (21 mL/ 16 mL) was added 0.65 mL of concentrated sulfuric acid and the mixture was heated under reflux overnight. The reaction mixture was cooled to room temperature and the methanol was removed under reduced pressure. The pH of the residue was adjusted to pH
10 by addition of saturated sodium carbonate solution and the resulting solution was extracted with chloroform (100 mL). The organic layer was washed with brine, dried over anhydrous sodium sulphate and reduced under pressure to afford a white solid (0.59 g, 33\%). $^1$H NMR (700MHz, CDCl$_3$) $\delta$ 8.05 (d, $J$ = 8.4 Hz, 1H), 7.90 (d, $J$ = 8.2 Hz, 1H), 7.69 (ddd, $J_1$ = 1.2, $J_2$ = 6.8, $J_3$ = 8.2 Hz, 1H), 7.51 (ddd, $J_1$ = 1.2, $J_2$ = 6.8, $J_3$ = 8.2 Hz, 1H), 7.50 (s, 1H), 5.20 (d, $J$ = 5.2 Hz, 2H), 2.75 (s, 3H). $^{13}$C NMR (175MHz, CDCl$_3$) $\delta$ 159.4, 147.9, 145.8, 129.5, 129.4, 124.2, 122.7, 119.2, 61.9, 25.6.

**Synthesis of 4-chloro-2-methylquinoline hydrochloride (5)**$^{29}$

To a solution of 4 (0.97 g, 5.6 mmol) in dry dichloromethane (10 mL/ 5 mL) was added thionyl chloride (0.81 mL, 11.2 mmol) dropwise at 0°C. After the addition was complete, the reaction was left to stir at room temperature for 15 hours. The reaction mixture was then concentrated under reduced pressure and the residue was washed with EtOAc and concentrated to obtain a light orange solid which was used with no further purification (1.15 g, 90%).

**Synthesis of 4-(methylsulfonyl) phenol (8)**

Copper (I) iodide (0.64 g, 3.2 mmol) was added to a pre-gassed solution of 4-iodophenol (2.3mmol, 0.50g) in DMF (7.5 mL). The mixture was purged with argon for 10 minutes and stirred at r.t for 1 minute. Sodium methanesulfinate (0.14 g, 2.3 mmol) was added, the reaction mixture purged with argon, sealed and heated at 110 °C for 72 hours. Following this, the mixture was diluted with water (40 mL) and
extracted with EtOAc (45 mL × 2). The organic phases were combined and washed with brine, dried over sodium sulphate and concentrated under reduced pressure. The crude oil was purified by flash chromatography (10% EtOAc/hexanes) and concentrated under reduced pressure to afford a white solid (0.36 g, 92%). $^1$H NMR (700MHz, DMSO-$d_6$) δ 10.56 (br s, 1H), 7.72 (d, $J = 8.7$ Hz, 2H), 6.94 (d, $J = 8.7$ Hz, 2H), 3.32 (s, 3H), 3.10 (s, 3 H). $^{13}$C NMR (175MHz, DMSO-$d_6$) 161.9, 130.8, 129.3, 115.7, 44.1.

**Synthesis of 2-methyl-4-((4-(methylsulfonyl)phenoxy)methyl)quinoline (9)**

Cesium carbonate (0.94 g, 2.9 mmol) was added to a solution of 4 (0.68 g, 2.9 mmol) and 7 (0.50 g, 2.9 mmol) in acetonitrile (25 mL). The solution was heated under reflux over night after which the reaction mixture was filtered and the solvent removed *in vacuo*. The crude was purified by flash chromatography (50% EtOAc/hexanes) and concentrated to afford a white solid (0.60 g, 64%). $^1$H NMR (700MHz, DMSO-$d_6$) δ 8.11 (d, $J = 7.7$ Hz, 1H), 7.98 (d, $J = 8.2$ Hz, 1H), 7.89 (d, $J = 8.9$ Hz, 2H), 7.75 (ddd, $J_1 = 1.2$, $J_2 = 6.8$, $J_3 = 8.2$ Hz, 1H), 7.59 (ddd, $J_1 = 1.2$, $J_2 = 6.8$, $J_3 = 8.2$ Hz, 2H), 7.38 (d, $J = 8.9$ Hz, 2H), 5.74 (s, 2H), 3.18 (s, 3H), 2.67 (s, 3H). $^{13}$C NMR (175MHz, DMSO-$d_6$) δ 161.8, 158.6, 147.4, 141.5, 133.2, 129.4, 129.3, 128.8, 125.9, 123.7, 120.2, 115.5, 66.7, 43.9, 24.9.

**Synthesis of methyl 5-methylnicotinate (11)**

5-methylnicotonic acid (80.0 mg, 0.6 mmol) was dissolved in 3 mL of methanol and thionyl chloride (0.09 mL, 1.2 mmol) was added slowly dropwise at 0°C. The
reaction was warmed to room temperature, and then refluxed for 18 hours. The ethanol was removed by evaporation and the residue was diluted with DCM (7 mL). The pH was adjusted to 10 with aqueous 1M NaOH and the resulting solution was extracted with ether (10 mL). The organic layer was washed with brine (10 mL) and dried over anhydrous MgSO₄. The solvent was removed under pressure and the crude mixture was purified by flash chromatography (40% EtOAc/hexanes) to afford a colourless oil (58.0 mg, 64%). $^1$H NMR (700 MHz, CDCl₃) $\delta$ 9.04 (s, 1H), 8.61 (s, 1H), 8.11 (s, 1H), 3.94 (s, 3H), 2.40 (s, 3H), $^{13}$C NMR (175 MHz, CDCl₃) $\delta$ 166.1, 153.9, 148.0, 137.7, 133.3, 125.8, 52.5, 18.4.

**Synthesis of 1-(5-methylpyridin-3-yl)-2-(4-((2-methylquinolin-4-yl)methoxy)phenyl)sulfonyl)ethanone (12)**

To a 50 mL oven dried round bottom flask was added 8 (70.0 mg, 0.2 mmol) and the flask was evacuated and backfilled with argon several times. The solid was dissolved in dry THF (5 mL) and the flask was cooled to -78°C by means of a dry ice bath. LDA (2.0 M in THF/heptane/ethylbenzene, 0.21 mL, 0.4 mmol) was added via a syringe drop wise under argon. After the addition, the mixture was left to stir for 1 hour at -78°C after which a solution of 11 (70.0 mg, 0.4 mmol) in dry THF (5 mL) was added drop wise under argon at this temperature. The reaction was then stirred for an additional hour at room temperature after which it was quenched with water (10mL) and extracted with EtOAc (10 mL × 2). The organic phase was dried over anhydrous MgSO₄, concentrated in vacuo and the residue was purified by
flash chromatography (5% MeOH/DCM) to afford a bright orange solid (57.0 mg, 64%). HRMS (ESI+) for calculated for C$_{25}$H$_{22}$N$_2$O$_4$S 447.1379, found: 447.1379.

**Synthesis of 1-(5-methylpyridin-3-yl)-2-(4-((2-methylquinolin-4-yl)methoxy)phenylsulfonyl)ethanol (14)**

To a solution of 12 (0.12 g, 0.28 mmol) in 12 mL methanol was added NaBH$_4$ (0.02 g, 0.56 mmol) at 0°C. The reaction was warmed to room temperature and stirred for 2 hours after which it was quenched with acetone and the solvent was removed under vacuum. The residue was purified by flash chromatography (10% MeOH/DCM) to furnish 14 as a white solid (0.10 g, 84%).

**Synthesis of N-hydroxy-N-(1-(5-methylpyridin-3-yl)-2-(4-((2-methylquinolin-4-yl)methoxy)phenylsulfonyl) ethyl) formamide (1)**

To a solution of 14 (30.0 mg, 0.07 mmol) in THF (2 mL) was added NH$_4$OH (50% wt. in water, 0.5 mL) and the reaction was refluxed at 80°C for 2 hours. The THF was evaporated and the residue was diluted with water (10 mL) and extracted with EtOAc (12 mL ×2). The organic phase was dried over anhydrous MgSO$_4$ and concentrated to give a white solid. The hydroxylamine was used in the next step without further purification.

To a solution of the hydroxylamine in MTBE (2 mL) was added 2,2,2-trifluoroethylformate$^{20}$ (0.04 mL, 0.4 mmol). The reaction was refluxed for 16 hours after which the MTBE was removed by rotary evaporation. The residue was purified by reverse phase C-18 column chromatography to afford a white solid (6.0 mg, 22%).
Synthesis of ethyl 2-(2H-pyran-4(3H,5H,6H)-ylidene)acetate (20a)

To a suspension of sodium t-butoxide (0.67 g, 7.04 mmol) in dry THF was added triethylphosphonoacetate (1.26 mL, 6.4 mmol) dropwise at 0°C. After the addition was complete, the mixture was stirred for 30 minutes at room temperature after which 18 (6.40 mmol, 0.6mL) in 4mL dry THF was added dropwise. The reaction was left to stir at room temperature for 16 hours. The reaction mixture was quenched with water and extracted with EtOAc (35 mL × 2). The organic phase was dried over anhydrous MgSO₄ and the solvent was removed under pressure. The crude was purified by flash chromatography to give a light yellow oil (0.40 g, 82%). ¹H NMR (700 MHz, CDCl₃) δ 5.68 (s, 1H), 4.15 (q, J = 7.1 Hz, 2H), 3.76 (m, 2H), 3.72 (m, 2H), 2.99 (m, 2H), 2.32 (m, 2H), 1.27 (m, 3H). ¹³C NMR (175 MHz, CDCl₃) δ 166.6, 157.3, 114.7, 69.2, 68.6, 59.9, 37.7, 31.2, 14.4.

Synthesis of tert-butyl 4-(2-ethoxy-2-oxoethylidene)piperidine-1-carboxylate (20b) was prepared as above to afford a white solid (0.98 g, 57%). ¹H NMR (700MHz, CDCl₃) δ 5.69 (s, 1H), 4.14 (q, J = 7.0 Hz, 2H), 3.46 (m, 4H), 2.26 (m, 2H), 1.46 (s, 9H), 1.26 (t, J = 7.0 Hz, 3H). ¹³C NMR (175MHz, CDCl₃) δ 166.4, 157.9, 154.7, 79.9, 59.9, 36.5, 29.6, 28.5, 14.4.

Synthesis of methyl 4-hydroxybenzoate (17a)

To a 50 mL round bottom flask was added 4-hydroxybenzoic acid (2.00 g, 14.5 mmol) in methanol (13 mL). To this solution was added concentrated sulfuric acid (2 mL) and the reaction was refluxed overnight. The mixture was allowed to cool
to room temperature and Na$_2$CO$_3$ was added to neutralize the reaction mixture. The methanol was removed under reduced pressure and the residue was taken up in dichloromethane (100 mL) and extracted with water (70 mL × 3). The organic phase was dried under anhydrous MgSO$_4$ and concentrated to give a white solid (2.09 g, 95%). $^1$H NMR (700MHz, CDCl$_3$) δ 7.95 (d, $J = 8.5$ Hz, 2H), 6.86 (d, $J = 8.6$ Hz, 2H), 5.26 (br s, 1H), 3.89 (s, 3H).$^{13}$C NMR (175MHz, CDCl$_3$) δ 167.1, 159.9, 132.1, 122.9, 115.3, 52.1.

**Synthesis of methyl-3-iodo-4-(2-methylquinolin-4-yl)methoxy)benzoate (17b)**

Prepared in accordance with the method described for the synthesis of 17a. (0.41 g, 95%) $^1$H NMR (700MHz,CDCl$_3$) δ 8.50 (s, 1H), 8.10 (d, $J = 8.3$ Hz, 1H), 8.10 (m, 1H), 8.02 (d, $J = 8.5$ Hz, 1H), 7.64 (s, 1H), 7.56 (m, 1H), 6.94 (d, $J = 8.61$ Hz, 2H), 5.63 (s, 2H), 3.90 (s, 3H), 2.78 (s, 3H).$^{13}$C NMR (175MHz, CDCl$_3$) δ 141.5, 131.80, 123.8, 122.53, 116.5, 111.5, 86.0, 52.4, 29.8. HRMS (ESI$^+$) for C$_{19}$H$_{16}$INO$_3$ Calcd. 434.0253, Obsd. 434.0240.

**Synthesis of methyl 2-(4-aminotetrahydro-2H-pyran-4-yl)acetate (21a)**

7.0M NH$_3$/MeOH (2 mL) was added via a syringe to a vial containing 19 (0.10 g, 0.67 mmol). The vial was sealed and heated at 80°C for 24 hours. The mixture was left to cool to room temperature and the solvent was removed by rotary evaporation. The residue was passed through a plug of silica and eluted in 10% MeOH/DCM. The filtrate was concentrated to afford a light yellow oil which was used with no further purification (49.0 mg, 42%)
Synthesis of 4-((2-methylquinolin-4-yl)methoxy)benzoic acid (18a)\textsuperscript{15}

Ester 17 (0.28 g, 0.9 mmol) was diluted in a 1:1 THF/1 M NaOH solution (3 mL) and heated at 60°C for 16 hours. The mixture was cooled and acidified using an aqueous 1 M HCl solution. The resulting white precipitate formed was filtered off, washed with water and dried in dessicator overnight. The resulting carboxylic acid 18a was used with no further purification. (0.23 g, 89%).

Synthesis of 2-(4-(3-iodo-4-((2-methylquinolin-4-yl)methoxy)benzamido) tetrahydro-2H-pyran-4-yl)acetic acid (18b)

Prepared in accordance with procedure described for synthesis of 18a. (50.0 mg, 90%) \textsuperscript{1}H NMR (700 MHz, MeOH-d4) δ 8.45 (d, J = 8.5 Hz, 1H), 8.32 (d, J = 8.5 Hz, 1H), 8.29 (s, 1H), 8.23 (d, J = 8.5 Hz, 1H), 8.11 (m, 1H), 7.94 (m, 1H), 7.92 (m, 1H), 7.35 (d, J= 8.5 Hz, 1H), 6.00 (s, 2H), 3.78 (m, 2H), 3.71 (m, 2H), 3.03 (s, 3H), 2.94 (s, 2H), 2.46 (d, J = 13.6 Hz, 2H), 1.84 (m, 2H), \textsuperscript{13}C NMR (175MHz, MeOH-d4) δ 174.1, 169.1, 160.1, 159.4, 140.0, 132.3, 130.7, 125.8, 125.5, 122.0, 113.2, 86.3, 68.6, 64.7, 54.0, 42.9, 35.9, 30.7, 21.8. HRMS (ESI\textsuperscript{+}) for C\textsubscript{25}H\textsubscript{25}IN\textsubscript{2}O\textsubscript{5}: Calcd. 561.0886, Obsd. 561.0886.

Synthesis of methyl2-(4-(4-((2-methylquinolin-4-yl)methoxy)benzamido) tetra hydro-2H-pyran-4-yl)acetate (22a)

To a solution of 18a (0.13 g, 0.44 mmol) in DMF (2 mL) was added PYBOP (0.23 g 0.44 mmol) and DIPEA (0.15 mL, 0.88 mmol) at 0°C. After 5 minutes of stirring at this temperature, a solution of the amine 21a (80.0 mg, 0.44 mmol) in DMF (1
mL) was added via syringe. The reaction was stirred at 0°C for 15 minutes and then warmed to room temperature where it was left stirring for 18 hours after which it was diluted with water and extracted with EtOAc (15 mL × 3). The organic layers were combined, dried over anhydrous MgSO₄ and concentrated in vacuo. The residue was purified by silica gel column chromatography (75% EtOAc/ hexanes) and the fractions were concentrated to afford 21 as a white solid (0.16 g, 83%). ¹H NMR (700 MHz, MeOH-d₄) δ 8.12 (d, J = 7.8 Hz, 1H), 8.01 (d, J = 8.3 Hz, 1H), 7.79 (dd, J = 8.8 Hz, 2H), 7.78 (ddd, J₁ = 1.2, J₂ = 6.8, J₃ = 8.3 Hz, 1H), 7.62 (m, 2H), 7.17 (d, J = 8.8 Hz, 2H), 5.69 (s, 2H), 3.77 (m, 2H), 3.72 (m, 2H), 2.73 (s, 3H), 2.97 (s, 2H), 2.42 (d, J = 12.9 Hz, 2H), 1.82 (m, 2H). ¹³C NMR (175 MHz, MeOH-d₄) δ 172.6, 170.8, 160.4, 160.3, 148.5, 144.8, 144.8, 131.0, 129.0, 127.5, 125.6, 124.5, 121.5, 120.4, 120.0, 115.6, 67.8, 53.9, 51.9, 43.2, 24.7.

Synthesis of 22b and 22c as described for the synthesis of 22a: (0.15 g, 65%). ¹H NMR (700 MHz, MeOH-d₄) δ 8.12 (d, J = 7.8 Hz, 1H), 8.01 (d, J = 8.3 Hz, 1H), 7.79 (dd, J = 8.8 Hz, 2H), 7.78 (ddd, J₁ = 1.2, J₂ = 6.9, J₃ = 8.3 Hz, 1H), 7.62 (m, 2H), 7.17 (d, J = 8.8 Hz, 2H), 5.69 (s, 2H), 3.77 (m, 2H), 3.72 (m, 2H), 2.97 (s, 2H), 2.73 (s, 3H), 2.42 (d, J = 13.0 Hz, 2H), 1.80-1.83 (m, 2H). ¹³C NMR (175 MHz, MeOH-d₄) δ 172.6, 170.8, 160.4, 160.3, 148.5, 144.8, 144.8, 131.0, 129.0, 127.5, 125.6, 124.5, 121.5, 120.4, 120.0, 115.6, 67.8, 53.9, 51.9, 43.2, 24.7.

Synthesis of 2-(4-(4-((2-methylquinolin-4-yl)methoxy)benzamido)tetrahydro-2H-pyran-4-yl)acetic acid (23a)
Ph.D. Thesis – Salma A. Al-Karmi; McMaster University – Chemistry & Chemical Biology.

Prepared in accordance with the method described for hydrolysis of 18. $^1$H NMR (700 MHz, MeOH-d4) $\delta$ 8.43 (d, $J = 8.5$ Hz, 2H), 8.17 (d, $J = 8.5$ Hz, 1H), 8.12 (m, 2H), 7.94 (m, 4H), 7.85 (d, $J = 8.8$ Hz, 2H), 7.25 (d, $J = 8.8$ Hz, 2H), 5.93 (s, 2H), 3.78 (m, 2H), 3.72 (m, 2H), 2.99 (s, 3H), 2.95 (s, 2H), 2.46 (d, $J = 13.4$ Hz, 2H), 1.83 (m, 2H). $^{13}$C NMR (175 MHz, MeOH-d4) $\delta$ 174.1, 170.5, 130.6, 130.5, 130.4, 125.7, 125.6, 121.6, 115.7, 67.3, 64.6, 53.8, 42.9, 35.9, 21.5.

**Synthesis of 2-(4-(3-iodo-4-((2-methylquinolin-4-yl)methoxy)benzamido) tetrahydro -2H-pyran-4-yl) acetic acid (23b)**

Prepared in accordance with procedure described for synthesis of 17. (50.0 mg, 90%) $^1$H NMR (700 MHz, MeOH-d4) $\delta$ 8.45 (d, $J = 8.5$ Hz, 1H), 8.32 (d, $J = 8.5$, 1H), 8.29 (s, 1H), 8.23 (d, $J = 8.5$ Hz, 1H), 8.11 (m, 1H), 7.94 (m, 1H), 7.92 (m, 1H), 7.35 (d, $J = 8.5$ Hz, 1H), 6.00 (s, 2H), 3.78 (m, 2H), 3.71 (m, 2H), 3.03 (s, 3H), 2.94 (s, 2H), 2.46 (d, $J = 13.6$ Hz, 2H), 1.85 (m, 2H). $^{13}$C NMR (175 MHz, MeOH-d4) $\delta$ 174.1, 169.1, 160.1, 159.4, 140.0, 132.3, 130.7, 125.8, 125.5, 122.0, 113.2, 86.3, 68.6, 64.7, 54.0, 42.9, 35.9, 30.7, 21.8. HRMS (ESI$^+$) for C$_{25}$H$_{25}$IN$_2$O$_5$ calcd. 561.0886, Obsd. 561.0886.

**Synthesis of N-(4-(2-(hydroxyamino)-2-oxoethyl)tetrahydro-2H-pyran-4-yl)-4-((2-methylquinolin-4-yl)methoxy)benzamide (2a)**

**Method 1:**

Carboxylic acid 23a (90.0 mg, 0.2 mmol) was suspended in DMF (1.5 mL) and to this was added HOBT. H$_2$O (30.0 mg, 0.2 mmol), NH$_2$-OTBDMS (14.0 mg, 0.2 mmol). The solution was cooled to 0°C and EDC.HCl (40.0 mg, 0.2mmol) was added slowly to the stirring mixture. The reaction was left stirring at this
temperature for 15 minutes and then warmed to room temperature. After 18 hours
the mixture was diluted with water (2 mL) and extracted with EtOAc (3 mL × 3).
The organic layers were combined, dried over anhydrous MgSO₄ and the solvent
was removed by rotary evaporation. The resulting residue was resuspended in a
minimum amount of water/acetonitrile and purified by means of a reverse phase
C18 column (provided by Teledyne Technologies inc., RediSep® Rf, 5.5g) (43%
acetonitrile/ water) to afford the free hydroxamic acid as a white solid (89.0 mg,
48%).
Method 2:
Compound 22a (43.0 mg, 0.09mmol) was added to a microwave vial equipped with
a stir bar and diluted in 1,4-dioxane (0.5 mL) and an equal volume of 50% aqueous
hydroxylamine solution. The vial was capped and subjected to microwave
irradiation (60°C, 150W) for 30 minutes. The reaction was diluted with EtOAc (2
mL) and washed with water. The organic layer was dried over anhydrous
magnesium sulfate and the solvent was removed by rotary evaporation. The
residue was purified in the manner described in method 1. (10.0 mg, 25%). ¹H NMR
(700 MHz, MeOH-d4) δ 8.12 (d, J = 7.9 Hz, 1H), 8.01 (d, J = 8.4 Hz, 1H), 7.82, (d,
J = 8.8 Hz, 2H), 7.77 (m, 1H), 7.61 (m, 2H), 7.16 (d, J = 8.8 Hz, 2H), 5.69 (s, 2H),
3.76 (m, 2H), 3.72 (m, 2H), 2.72 (s, 2H), 2.69 (s, 2H), 2.47 (d, J = 13.6 Hz, 2H),
1.82 (m, 2H). ¹³C NMR (175 MHz, MeOH-d4) δ 170.6, 169.4, 162.3, 160.4, 148.5,
144.8, 131.0, 130.5, 130.0, 129.0, 127.5, 124.5, 121.6, 115.6, 67.8, 64.7, 54.3,
41.5, 35.9, 28.7, 24.7. HRMS (ESI⁺) for C₂₅H₂₈N₃O₅ Calcd.,450.2029, Obsd.

- 153 -
450.2026. 2b and 2c prepared in accordance with method 1 for synthesis of 2a. 

2b: (4.0 mg, 57%). 1H NMR (700 MHz, MeOH-d4) δ 8.31 (d, J = 2.1 Hz, 1H), 8.15 (d, J = 8.3 Hz, 1H), 8.02 (d, J = 8.4 Hz, 1H), 7.88 (d, J = 2.1 Hz, 1H), 7.87 (d, J = 2.1 Hz, 1H), 7.80 (s, 1H), 7.79 (dd, J1 = 1.3, J2 = 6.9, J3 = 8.3 Hz, 1H), 7.64 (m, 1H), 7.24 (d, J = 8.6 Hz, 1H), 5.77 (s, 2H), 3.76 (m, 2H), 3.70 (m, 2H), 2.75 (s, 3H), 2.68 (s, 2H), 2.46 (d, J = 13.6 Hz, 2H), 1.82 (m, 2H). 13C NMR (175 MHz, MeOH-d4) δ 169.4, 169.1, 160.6, 160.4, 148.4, 144.4, 140.1, 131.8, 131.1, 130.5, 129.0, 127.6, 125.4, 124.6, 121.6, 112.9, 86.4, 68.8, 64.7, 54.5, 41.4, 35.9, 24.9. HRMS (ESI+) for C25H26N3O5 Calcd. 576.0995 Obsd. 576.0972

2c: 1H NMR (600 MHz, DMSO-d6) δ 8.12 (d, J = 8.3 Hz, 2H), 7.97 (d, J = 8.3 Hz, 1H), 7.81 (d, J = 8.7 Hz, 2H), 7.78 (m, 1H), 7.58 (m, 2H), 7.19 (d, J = 8.7 Hz, 2H), 5.68 (s, 2H), 4.53 (m, 1H), 4.46 (m, 1H), 2.73 (s, 3H), 2.66 (s, 3H), 2.55 (m, 4H), 2.44 (d, J = 12.1 Hz, 2H), 2.28 (m, 2H), 1.74 (s, 1H), 1.62 (m, 2H). 13C NMR (150 MHz, DMSO-d6) 166.4, 166.0, 158.6, 147.4, 141.9, 129.4, 129.3, 128.8, 125.9, 123.8, 123.7, 120.1, 114.2, 82.4, 81.5, 66.3, 57.7, 57.6, 52.8, 49.4, 33.1, 24.9. HRMS (ESI+) for C27H32FN4O4 Calcd. 495.2408 Obsd. 495.2408

5.6 References


Chapter 6: Conclusions and Future Work

The current diagnostic imaging methods used in clinical practice to identify and characterize RA are severely limited. Radiography is the most widely used imaging modality but is generally most effective for late-stage disease where there is bone erosion and deformities.¹,² Existing medications in contrast are most effective when they are used to treat this disease at an early stage.³ Molecular imaging can be used not only as a diagnostic tool, but also as a means to monitor therapeutic response and aid in drug discovery. For this reason, the development of molecular imaging probes for RA can directly impact patient prognosis by aiding in therapeutic treatment plans and decreasing the severe side-effects often associated with RA treatment.

This thesis describes three strategies towards molecular imaging of RA. The first approach involves the development of a hybrid optical-PET probe that responds to ROS. This probe is currently the first reported example of a NIR turn-on optical-PET probe (Chapter 2). The PET agent exhibited a biodistribution pattern reminiscent of albumin-related blood-pool imaging agents, which are capable of accumulating in tissues exhibiting hypervascularity. The fluorescent counterpart is significantly more stable than the commonly employed ROS probe DHE and has been shown to specifically react with superoxide and hydroxyl radicals. Such a probe would be valuable not only in assessing RA, but also for cancer and cardiac ischemia.
The second approach involves the development of NIR fluorinated fluorophores that were encapsulated in F-127 micelles. One of the synthesized fluorophores is a meso-fluorinated dye that was prepared via a HALEX reaction. This fluorophore is the first example of a meso fluorinated cyanine dye that shows enhanced fluorescence response both in solution and in a PC-3 cell assay in comparison to the commercially available chlorinated analogue (IR780). The micelle encapsulated fluorophores also show enhanced fluorescence in the encapsulated micelles and are completely soluble in aqueous buffer. Stability studies show no evidence of aggregation in solution up to two weeks which is advantageous for general use and longer term storage of the agent. These micelles have the ability to localize in hypervascular diseased tissue found in RA where the fluorophore is expected to accumulate based on the EPR effect.

The third approach involved the development of a targeted probe that binds to the metalloprotease TACE. This enzyme is heavily involved in RA (and other diseases such as cancer) initiation and progression. The preliminary data presented describes the synthesis and in vitro evaluation of two hydroxamic acids, one of which exhibits an average IC$_{50}$ of 10 nM. Given the success of the work on the ROS probes this work was not advanced beyond development and screening of the lead candidates.

6.1 Future work and Preliminary Results

The immediate future work is to evaluate the ROS probes developed during the course of this work in different preclinical models. In particular evaluation of the
micelle entrapped dyes appears to be the most viable route to achieving the desired target- to-non-target ratio. For the albumin binding probe, work should focus on evaluating the compound in vivo compared to labeled albumin or other albumin binding agents including $^{18}$F Evan’s blue, which was recently reported.\(^4\)

An additional avenue of future research is to prepare more water soluble $^{18}$F-labeled cyanine and hydrocyanine dyes. The $^{18}$F labeling of cyanine fluorophores is challenging due to the sensitive nature of the dye to the typically harsh reaction conditions used during labeling. Further, the labeling of ROS-sensitive probes is made more difficult by stability issues arising from radiation-induced radical formation in solution. Currently, there are two known examples of $^{18}$F cyanine dyes: one based on isotopic exchange at the center of a “boron trap”,\(^5\) and the other which employed a pre-labeled prosthetic group.\(^6\) Through the work described in this thesis, it has been shown that the introduction of the o-nitropyridine functionality onto commercially available meso-chlorinated dyes can be accomplished by a robust Suzuki coupling reaction.\(^{18}$F labeling can be achieved in good yield in one step by heating the precursors for a very short period of time (4 minutes). To assess whether this was translatable to other structurally similar dyes, the same procedures were used to prepare a related probe derived from IR775 chloride (Chapter 4), resulting in comparable RCYs. Given that minor structural variations on cyanine fluorophores have resulted in dramatic differences in biodistribution patterns,\(^7,8\) detailed exploration of various structural analogues in a number of preclinical models is required (Figure 6.1). Further, given the blood
binding ability of the first generation ROS probe, this probe can be assessed in a model that assesses lymphatic drainage to determine whether it can be useful in lymph node mapping.

Figure 6.1 Structural modifications that can be made on the cyanine dye scaffold for assessment in preclinical models.

While there are imaging agents targeted at MMP enzymes\(^9\) and the related cytokine TNF-\(\alpha\)\(^10\), the enzyme TACE has not been targeted to date for molecular imaging purposes. One challenge may be that the current known small-molecule TACE inhibitors possess sensitive hydroxamic acid functionalities that are prone to hydrolyze/degrade \textit{in vivo} or under certain reaction conditions.\(^11\) Consequently, \(^{18}\text{F}\) labeling of compounds possessing this functionality may be more efficiently achieved \textit{via} a prosthetic group approach. The azide-alkyne Huisgen cycloaddition\(^12\) is commonly exploited in \(^{18}\text{F}\) labeling reactions due to the rapid and facile nature of the reaction. Ester 2 was synthesized as an intermediate for the
purpose of generating the corresponding hydroxamic acid 4a. It has been previously demonstrated that functionalization at the piperidine nitrogen of this scaffold is tolerable in that inhibitory activity is not drastically altered.\(^\text{13}\) 4a must first be assessed for activity in an in vitro assay to confirm that the triazole ring does not negatively impact inhibitory activity.

Scheme 6.1 Synthesis of precursors for the synthesis of potential TACE inhibitor 4. i) Propargyl bromide, NaH, THF, r.t, 30 min, 66%. ii) 1-azido-2-fluoroethane, sodium ascorbate, Cu (II) acetate, DMF, r.t., 1 hour, 36%.

Scheme 6.2 Prosthetic group radiolabeling strategy for the synthesis of 4b. a) Synthesis of \(^{18}\text{F}\)-ethyl azide i) K \([^{18}\text{F}]\), K\(_2\)CO\(_3\), K\(_{222}\). b) Conjugation of radiolabeled prosthetic group via the click reaction ii) Cu(II) catalyst, sodium ascorbate.
$^{18}$F labeling can be performed by first synthesizing the tosylate 5 and then subjecting this to conventional nucleophilic fluorination conditions. The product 6 can be reacted with intermediate 7 to generate the anticipated product. As a proof of concept, tracer 4b can be assessed in various tumour cell lines, such as MCF-7 which are known to show enhanced TACE activity.\textsuperscript{14}

6.2 Experimental

Synthesis of 2

Compound 1 (0.11 g, 0.25 mmol) was dissolved in dry THF (4 mL) and carefully added to an oven-dried 25 mL flask containing a slurry of NaH (60% dispersion in mineral oil, 70.0 mg, 1.7 mmol) in THF (4 mL). Propargyl bromide (80% wt solution in toluene, 0.15 mL, 1.0 mmol) was slowly added to the mixture via a syringe and after addition, the mixture was stirred at r.t for 30 min. The reaction was quenched with brine and extracted with EtOAc ($2 \times 20$ mL). The organic phases were combined, dried over anhydrous MgSO$_4$ and concentrated \textit{in vacuo}. The desired product was isolated using a 4 gram flash RediSep® Rf silica gel column (30% MeOH/DCM) to afford 2 as a white solid (80.0 mg, 66%). $^1$H NMR (700 MHz, MeOH-d4): δ 8.10 (d, $J = 8.5$ Hz, 1H), 8.00 (d, $J = 8.5$ Hz, 1H), 7.78, (d, $J = 8.7$ Hz, 2H), 7.76 (m, 1H), 7.60 (m, 2H), 7.16 (d, $J = 8.7$ Hz, 2H), 5.69 (s, 2H), 3.60 (s, 2H), 3.35 (s,3H), 3.31 (m, 4H), 2.93 (s, 1H), 2.76 (d, $J = 12.3$ Hz, 2H), 2.62 (t, $J = 11.4$ Hz, 2H), 1.95 (s, 3H), 2.54 (d, $J = 12.3$ Hz, 2H), 1.82 (m, 2H), $^{13}$C NMR (175 MHz, MeOH-d4) δ 172.6, 170.7, 162.3, 160.4, 148.5, 144.8,
131.0, 130.4, 130.1, 129.0, 127.5, 125.6, 124.5, 121.5, 115.6, 78.9, 75.5, 67.7, 53.9, 51.9, 47.3, 34.7, 24.7. HRMS (ESI\(^+\)) for \(\text{C}_{29}\text{H}_{32}\text{N}_{3}\text{O}_{4}\) Calcd. 486.2393 Obsd. 486.2417.

6.3 References


APPENDIX 1

Figures:

Figure S1 $^1$H NMR spectrum of 2 (700 MHz, MeOH-d4)
Figure S1-1 Expansion of the $^1$H NMR of 2 (700 MHz, MeOH-d4)

Figure S1-2 Expansion of the $^1$H NMR of 2 (700 MHz, MeOH-d4)
Figure S2. $^{13}$C NMR spectrum of 2 (150 MHz, MeOH-d4)
**Figure S2-1.** Expansion of the $^{13}$C NMR spectrum of 2 (150 MHz, MeOH-d4)

![NMR spectrum of 2](image)

**Figure S2-2.** Expansion of the $^{13}$C NMR spectrum of 2 (150 MHz, MeOH-d4)

![NMR spectrum of 2](image)
Figure S3. COSY spectrum of 2 (700 MHz, MeOH-d4)

Figure S4. HRMS of 2 (ESI⁺)
**Figure S5.** $^1$H NMR spectrum of 1a (700 MHz, CD$_3$CN)
Figure S5-1. Expansion of the $^1$H NMR spectrum of 1a (700 MHz, CD$_3$CN)

Figure S5-2. Expansion of the $^1$H NMR spectrum of 1a (700 MHz, CD$_3$CN)
Figure S6. $^{13}$C NMR spectrum of 1a (150 MHz, CD$_3$CN)
Figure S6-1. Expansion of the $^{13}$C NMR spectrum of 1a (150 MHz, CD$_3$CN)

Figure S6-2. Expansion of the $^{13}$C NMR spectrum of 1a (150 MHz, CD$_3$CN)
Figure S7. COSY spectrum of 1a (700 MHz, CD$_3$CN)

Figure S8. HRMS of 1a (ESI$^+$)
Figure S9. Stability profile of 1a ($\lambda_{ex}/\lambda_{em} = 768$ nm/794 nm) and dihydroethidium (DHE, $\lambda_{ex}/\lambda_{em} = 515$ nm/560 nm) at a concentration of 40 µM in 20% MeOH/PBS (pH= 7.4). Probes were incubated at 37°C and fluorescence readings were taken at the specified time intervals.

![Graph showing fluorescence intensity over time for 1a and DHE](image)

Figure S10. UV HPLC ($\lambda = 254$ nm) chromatograms of 1a in 20% MeOH/PBS after incubation at 37°C at 1 and 6 h (Method B).

![UV HPLC chromatograms for 1a and DHE](image)
Figure S11. Fluorescence intensity of 1a, and DHE (50 µM in MeOH) in the absence (no induction) and presence (induction) of KO₂, after 1 h at RT.

Figure S12. Fluorescence microscopy images of PC-3 cells treated with a) 50 µM 1a (1% DMSO) for 0.5 h following treatment with 0.8% saline for 0.5 h b) 50 µM 1a (1% DMSO) for 0.5 h following treatment with 5 mM TEMPOL (0.8% saline) for 0.5 h c) control image following treatment with 0.8% saline for 1 h and 1% DMSO for 0.5 h d) 50 µM 1a (1% DMSO) for 0.5 h following treatment with 0.8% saline for 1 h e) 50 µM 1a following treatment with 5 mM NAC for 1h (f) Brightfield image. TEMPOL = 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl; NAC = N-acetyl-L-cysteine. Note that we found this assay highly variable.
Figure S13. MTT assay of PC-3 cell viability, with various concentrations of 1a or cisplatin.

Figure S14. ORTEP projection for 3 (50% thermal ellipsoid probability). Hydrogen atoms and counter ion were omitted for clarity.
Figure S15. $^1$H NMR spectrum of 3 (700 MHz, MeOH-d4)
Figure S15-1. Expansion of the $^1$H NMR spectrum of 3 (700 MHz, MeOH-d4)

Figure S15-2. Expansion of the $^1$H NMR spectrum of 3 (700 MHz, MeOH-d4)
Figure S16. $^{13}$C NMR spectrum of 3 (150 MHz, MeOH-d4)
Figure S16-1. Expansion of the $^{13}$C NMR spectrum of 3 (150 MHz, MeOH-d4)

Figure S16-2. Expansion of the $^{13}$C NMR spectrum of 3 (150 MHz, MeOH-d4)
Figure S17. COSY spectrum of 3 (700 MHz, MeOH-d4)

Figure S18. HRMS of 3 (ESI+)
Figure S19. γ-HPLC chromatograms following the incubation of 1b 10% ethanol/saline for 60 and 120 min (Method A).

Figure S20. γ-HPLC chromatograms following the incubation of 1b in 10% ethanol/saline in the presence of ascorbic acid (0.5 mg/mL) for 60 and 120 min (Method A).
Figure S21. Standard curve of fluorescence intensity versus concentration of 1a.

Figure S22. Reconstructed PET/CT images at 1, 2 and 4 h post intravenous injection of 1b in a healthy C57BL/6 mouse.
Tables:

Table S1. X-ray Crystallography Data for 3

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Table S2. Biodistribution of 1b after intravenous injection into healthy C57BL/6 mice, sacrificed at 2 and 4 h. Data is expressed in % injected dose/gram of tissue or fluid (%ID/g).

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APPENDIX 2

Figure S1. $^1$H NMR spectrum of 7 (700 MHz, MeOH-d4)
Figure S2. $^{13}$C NMR spectrum of 7 (175 MHz, MeOH-d4)
**Figure S3.** HRMS of 7 (ESI+)**

![HRMS of 7 (ESI+)](image-url)

**Elemental Composition**

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**Notes**

- For Help, press F1
Figure S4. $^1$H NMR spectrum of 9a (600 MHz, MeOH-d4)
Figure S5. $^{13}$C NMR spectrum of 9a (150 MHz, MeOH-d4)
Figure S6. HRMS of 9a (ESI+).
Figure S7. $^1$H NMR spectrum of 11 (600 MHz, MeOH-d4)
Figure S8. $^{13}$C NMR spectrum of 11 (150 MHz, MeOH-d4)
Figure S9. HRMS of 11 (ESI+).
Figure S10. $^1$H NMR spectrum of 13 (600 MHz, MeOH-d4)
Figure S11. $^{13}$C NMR spectrum of 13 (150 MHz, MeOH-d4)
Figure S12. HRMS analysis for 13 (ESI⁺).

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