MULTIMODALITY SOMATOSTATIN ANALOG FOR FLUORESCENCE-GUIDED SURGERY IN CANCER

Servando Hernandez Vargas

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MULTIMODALITY SOMATOSTATIN ANALOG FOR FLUORESCENCE-GUIDED SURGERY IN CANCER

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MULTIMODALITY SOMATOSTATIN ANALOG FOR FLUORESCENCE-GUIDED SURGERY IN CANCER

A

THESIS

Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

by

Servando Hernandez Vargas, B.S.

Houston, Texas

December, 2018
Dedication

This thesis is dedicated to my parents, Eglee and Giovanni, my sister, Amarú, and grandparents, Sara, Reina, Pablo and Servando.
Acknowledgments

My deepest and sincerest gratitude to my mentor and friend, Ali Azhdarinia, Ph.D., for guiding me and allowing me to further my academic, professional and personal growth in his laboratory. I have truly enjoyed our discussions and look forward to being involved in many more projects!

I want to thank my colleague and friend, Sukhen C. Ghosh, Ph.D., for sharing all his vast expertise and for his continuous encouragement. I also want to thank my colleagues and friends, Julie Voss and Jo Simien, members of the Azhdarinia laboratory, for their patience and support throughout the past 2 years.

My gratitude and appreciation to my advisory committee, Kendra S. Carmon, Ph.D., Laura Smith Callahan, Ph.D., Jeffrey A. Frost, Ph.D., John Rasmussen, Ph.D., and Zhen Fan, M.D., for their guidance and the valuable discussions that have greatly contributed to this work.

Last but not least, I want to sincerely thank the MD Anderson UTHealth Graduate School community, especially Eric Swindell, Ph.D., Lindsey Minter, Ph.D., Brenda Gaughan, Elisabet Lau, Bunny Perez and Lily D’Agostino, for their always-outstanding support.
Cancer surgery remains the primary curative treatment for most solid cancers and has major therapeutic implications for patients with neuroendocrine tumors (NETs). Anatomical and functional imaging technologies are widely used during the pre- and postoperative stages, but intraoperative disease recognition relies on direct visual inspection and the hands of surgeons. The limited number of clinical tools for real-time intraoperative visual feedback restricts the ability to remove the complete cancer source and is partially responsible for the high rate of disease recurrence in patients. Intraoperative imaging with fluorescent contrast agents has the potential to improve the ability of surgeons to detect tumors when compared to visual inspection and hands alone. Growing clinical evidence highlights the utility of fluorescence-guided surgery (FGS) in cancer. However, the translation of fluorescently labeled imaging agents has been limited by the semi-quantitative nature of the optical signal. Strategies to combine radioactive and fluorescent contrast have been developed to enable cross-validation of fluorescent agents with nuclear imaging and quantitative analysis. While several methods for dual labeling have been proposed, the selection of a clinical radiotracer as a model system provides a proven targeting approach. Since adding a fluorophore to a radiotracer could adversely affect its imaging properties, we developed a multimodality chelator (MMC) to synthesize a bioactive analog of the NET imaging agent, $^{68}$Ga-DOTA-TOC. The MMC serves as a “radioactive linker” to bridge the near-infrared fluorescent (NIRF) dye IR800 and targeting moiety Tyr-3-octreotide (TOC), producing MMC(IR800)-TOC. Here, we first examined the radiochemical and pharmacological properties of the dual-labeled analog. Subsequently, we evaluated the ability of the fluorescent somatostatin analog to selectively target tumors that overexpress the somatostatin receptor subtype-2 (SSTR2) and demonstrate utility for FGS. We used $^{67}$Ga/$^{68}$Ga for quantitative biodistribution
studies and cross-validated semi-quantitative findings from fluorescence-based detection methods. The observed receptor-mediated uptake in mice was confirmed via \textit{ex vivo} analysis at the macro-, meso- and microscopic levels. These results showed the impact of dual labeling on tracer validation and the effectiveness of the MMC technology for developing a novel FGS agent.
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CHAPTER 1: INTRODUCTION
The role of surgery in cancer

The significant role of cancer surgery in patient care continues to evolve alongside breakthroughs in medical and molecular oncology (1). Beyond being an isolated and radical specialty, surgical oncology also extends to cancer prevention, diagnosis, staging, risk-reduction and multidisciplinary management. Historically, surgery has served as the first-line therapy for risk- and symptom-reduction for most solid cancers, and can be curative if complete resection is achieved (1–5). However, the presence of positive surgical margins (PSMs) occur when the surgical procedure does not remove the entire tumor and cancerous cells are left behind (6–10). As outlined by the International Union Against Cancer (UICC), the extent of residual cancerous tissue serves as a prognostic factor and may determine patient outcomes (11, 12).

Several surgical procedures are routinely employed for debulking or relieving symptoms. However, the ultimate goal of cancer surgery is to remove the complete source of the disease while sparing healthy tissue. Anatomical and molecular imaging technologies are available during pre- and postoperative stages for surgical planning and surveillance, respectively. Conversely, intraoperative disease recognition relies on direct visual inspection by the surgeon along “hands-on” palpation and tactile-guidance (1). As a result, significant effort has been made to bridge preoperative imaging with real-time intraoperative imaging to address a critical unmet need in cancer surgery.

Challenges, unmet needs and new technologies in surgical oncology

The advent of minimally invasive and robotic-assisted surgeries have revolutionized the surgical standard of care and improved patient outcomes (1, 4, 5). Despite the surge of technological advances in the operating room (OR), surgeons continue to rely on palpation and visual inspection for the discrimination between diseased and healthy tissue. Lesions that are undetectable to the naked eye, i.e., occult or too small, further complicate the ability of surgeons to differentiate cancerous from healthy tissue intraoperatively. In this context, the available gold standard for the semi-real-time intraoperative evaluation of PSMs is based on histopathology.
(13, 14). However, frozen section analysis has inherent challenges that may require extensive sampling of the wound bed and adjacent areas. This labor- and time-intensive method can also lead to the unnecessary removal of physiological elements or the partial extraction of diseased tissue (13–15). Patients with residual cancer clusters left behind due to incomplete resections or inconspicuous micrometastases have been shown to have a high recurrence rate and worse prognosis (6).

To improve upon existing methods for intraoperative tumor identification, live navigation using light-emitting agents that color code the surgical field of view has emerged as a promising alternative (16–18). Enhancing intraoperative disease mapping via optical imaging is projected to cause a paradigm shift in surgical oncology. The benefits of using fluorescence-guided surgery (FGS) would not only support current guidelines focused on preservation of anatomical structures (7, 8), but would also complement rapidly expanding minimally-invasive and robotic technologies where tactile-guidance is not possible (1, 7, 8).

**The nature of biomedical optics and its role in clinical oncology**

The foundation of *in vivo* optical imaging is based on the movement of photons through tissues and their interaction with tissue elements. Living organisms have endogenous contrast that originates from many biological substances, which dictate the extent of photon scattering (attenuation), absorption and emission (autofluorescence) characteristics of the living system (19, 20). These dynamic phenomena have been used to produce visual contrast, thus generating an opportunity for the application of biomedical optics (21). Alternatively, fluorescent signal can be produced through administration of exogenous contrast agents. These agents have clinical utility and are generally categorized based on whether the photons emitted are in the visible electromagnetic spectrum (400-600 nm) or in the near-infrared (NIR) region (700-900 nm) (22, 23).

The first clinical use of a fluorophore for enhancing the real-time localization of tumors dates back to 1948 when surgeons used fluorescein ($\lambda_{ex}/\lambda_{em} = 494/512$ nm) to aid in the
localization and identification of intracranial neoplasms (24). The authors concluded that fluorescein proved useful in confirming the presence or absence of neoplastic tissue. Additionally, the authors suggested the contrast-enhancing technique as a method for evaluating the state of infiltrating gliomas during surgery. Since then, the clinical benefits of fluorophores have supported the expansion of food and drug administration (FDA)-approved visible fluorescent agents (Table 1) for tumor detection and sentinel lymph node (SLN) mapping, as well as others (18). However, the wide-spread clinical translation of visible fluorophores has been restricted by their relatively low maximum resolution and penetration depth. These limitations are a result of the complex optical properties of the dyes, predominantly a combination of autofluorescence and photon attenuation that are particularly amplified in the 400-600 nm range (21) (Fig. 1).


<table>
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<tr>
<th>Fluorescence probe</th>
<th>Excitation</th>
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<td>780 nm</td>
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<td>634 nm (acid pH)</td>
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<td>Fluorescein sodium</td>
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<td>495 nm (folate-FITC)</td>
<td>520 nm (folate-FITC)</td>
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<td>IRDye800CW conjugate</td>
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<td>796 nm</td>
<td>IRDye800</td>
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Figure 1. NIR fluorescence is more suitable for in vivo imaging applications than visible-light fluorescence. Near-infrared (NIR) fluorophores (700–900 nm) have deeper tissue penetration and lower background fluorescence than visible-light fluorescence, resulting in enhanced signal-to-noise ratios. The detection depths achievable with the currently available instrumentation ranges from millimeters with NIR fluorescence to micrometers with visible-range fluorescence. Figure as originally published with permission from Ray R. Zhang, Alexandra B. Schroeder, Joseph J. Grudzinski, Eben L. Rosenthal, Jason M. Warram et al. (2017). Nat Rev Clin Oncol. doi: 10.1038/nrclinonc.2016.212.
Fluorophores in the NIR spectrum provide several advantages that make long-wave imaging specifically suitable for clinical use. This includes enhanced photon transport that leads to higher penetration depths. It also results in lower background signal due to reduced autofluorescence and photon scattering (Fig. 1) (25). The increasing demand for NIR dyes has been accompanied by the commercialization of FDA-approved NIR imaging devices (Table 2), which consist of an excitation light source, collection optics (filters), a NIR camera and a display unit (Fig. 2) (26, 27). Customization of the acquisition features of the imaging device can result in improved resolution and sensitivity with reduced amounts of dye (28). Thus, the concomitant development of a drug intended for use with a specific device (drug-device combination) can strongly support translational efforts.

Thus far, the clinical adoption of NIF imaging has been restricted to the high-dose administration (up to 25 mg) of the non-targeted dye, indocyanine green (ICG, $\lambda_{ex}/\lambda_{em} = 785/830$ nm). ICG has been widely used for the assessment of blood flow and tissue perfusion, SLN mapping, and tumor margin delineation (29–31). Since ICG lacks functional groups for bioconjugation, there has been an increasing demand for new NIR beacons that can build upon its clinical success, while providing improved stability and optical properties (25, 32).
Figure 2. The mechanics of NIR fluorescence imaging. NIR fluorescent contrast agents are administered intravenously, topically or intraparenchymally. During surgery, the agent is visualized using an NIR fluorescence imaging system of the desired form factor (above the surgical field for open surgery, or encased within a fiberscope for minimally-invasive and robotic surgery). All systems must have adequate NIR excitation light, collection optics, filtration and a camera sensitive to NIR fluorescence emission light. An optimal imaging system includes simultaneous visible (white) light illumination of the surgical field, which can be merged with the generated NIR fluorescence images. The surgeon’s display can be one of several form factors, including a standard computer monitor, goggles or a wall projector. Current imaging systems operate at a sufficient working distance that enables the surgeon to operate and illuminates a sizable surgical field. Abbreviations: LED, light-emitting diode; NIR, near-infrared. Figure as originally published with permission from Alexander L. Vahrmeijer, Merlijn Hutteman, Joost R. van der Vorst, Cornelis J. H. van de Velde, John V. Frangioni. Nat. Rev. Clin. Oncol. 10, 507–518 (2013). doi: 10.1038/nrclinonc.2013.123.

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<th>PerkinElmer Solaris</th>
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<th>Quest Spectrum</th>
<th>Novadaq SPY Elite</th>
<th>Hamamatsu PDE Neo</th>
<th>VisionSense Iridium</th>
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<td>Compact, good sensitivity</td>
<td>Excellent optics, large range of FOV</td>
<td>Simultaneous white-light, multichannel fluorescence overlay</td>
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<td>Compact, low cost, sensitive to low concentrations and overlay capability</td>
<td>Sensitivity to low concentrations</td>
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<td>No white-light Commercial system imaging</td>
<td>Unavailable at the time of testing</td>
<td>Nonlinear</td>
<td>No white-light imaging, nonlinear</td>
<td>No white-light imaging, nonlinear</td>
<td>Single fluorescence channel on the commercial system</td>
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*VisionSense Iridium has an FDA-approved unit with 805-nm excitation laser. For the sensitivity test, a 785-nm laser was used, which VisionSense sells as a research-only option.

*Quest offers a research-only custom-designed option on the Spectrum system that has 12-bit raw data export capability, such a system designed to our specifications was unavailable at the time of writing this article, and all tests were performed using the FDA-approved version of the system.

*Total cost is inclusive of only the device initial cost, so that any yearly maintenance costs, license costs, and cost of disposables, if any, were not factored in. However, annual recurring service fee costs are conventionally 10% to 20% of the initial cost, and prices of disposables can vary widely between systems.

*Sensitivity test data from the SurgVision prototype system were performed not performed using the same methodology as others NA = information not available.
Strategies for targeted FGS agent development

Conjugating a fluorophore to a ligand that binds to a target overexpressed in cancer would generate a disease-specific probe. The targeted approach would, in turn, require reduced amounts of contrast agent (μg range), and potentially lower risks of toxicity and non-specific signal (33). In its simplest form, the anatomy of a receptor-specific agent is comprised of a dye attached to a targeting moiety via a standard chemical linker. Depending on the receptor of interest, the targeting ligand may be a small molecule, antibody, peptide or other type of molecule. To facilitate the development of targeted probes, fluorophores have been synthesized to contain reactive groups for facile bioconjugation, i.e., via amine-reactive (i.e., NHS-ester) or sulfhydryl-reactive (i.e., maleimides) crosslinker chemistry, copper-free click chemistry (i.e., DBCO), etc. IR800 (λ_{ex}/λ_{em} = 774/789 nm) has emerged as a preferred bioconjugatable NIRF dye that positions as an excellent candidate for FDA-approval based on desirable optical properties, robust in vivo photostability and demonstrated safety both pre-clinically and clinically (17, 25, 34, 35).

Seminal first-in-human studies have shown the feasibility of utilizing targeted agents for FGS in several cancers. Notably, these targeted optical agents have proven to significantly enhance the identification/delineation of tumor deposits and increase resection-rates of malignant tissue when compared to palpation and visual inspection alone. Van Dam et al. reported the first clinical study with a targeted fluorophore and showcased the potential for improved cytoreductive surgery (36). This successful first-in-human application used a folate analog conjugated to fluorescein isothiocyanate (folate-FITC, λ_{ex}/λ_{em} = 495/520 nm) for tumor-specific intraoperative imaging of folate receptor-α (FR-α) overexpression. The subsequent rapid expansion of targeted imaging probes in the NIR region is exemplified by the development of OTL38, a folate analog conjugated to a NIRF dye (796 nm). OTL38 has been reported to enable higher detection rates of malignant tissue during surgery in FR-α positive malignancies including ovarian cancer (37), renal cell carcinoma (38), lung/pleural nodules (39) and others. Recently, IR800 has been conjugated to clinically used antibody therapeutics, such as bevacizumab (40).
and cetuximab (34), for successful receptor-mediated surgical navigation and tumor margin assessment in breast cancer and metastatic head and neck cancer patients, respectively.

While the utility of FGS in cancer continues to be supported by growing clinical evidence, the pathway for the successful clinical translation of optical probes is vague (41–45). The laboratory-to-human translation of targeted imaging probes continues to be largely restricted by the complexity of biomedical optics, the lack of a clinical benchmark and validation difficulties posed by the semi-quantitative nature of the optical signal (17, 21). Efforts to address FDA requirements have more often than not been particularly hampered by the lack of robust and standardized validation methods. The general consensus is that in order for image-guided surgery with fluorescent contrast agents to go from bench-to-bedside, it is fundamental to set standards with defined metrics for reporting and quantification in FGS (46–48). These realities have spurred increased interaction between surgeons, scientists and regulatory agencies to critically assess the value of implementing the optical technology in the OR and to identify optimal routes for its safe and efficacious translation (41–45).

**Dual labeling as a validation strategy**

It has been estimated that ~10% of the successful dye excitation events typically emit radiation (49). The intrinsic low energy radiated photons are then highly scattered in tissue, leading to signal loss and restricted depth of penetration, which significantly limits the acquisition efficiency by the optical imaging device. This scattering phenomenon largely contributes to the ambiguity associated with precisely measuring the fluorescent signal being produced (46–48). To overcome these quantitative limitations, efforts towards the combination of NIRF (>750 nm) and nuclear contrast into single, molecularly targeted agents have emerged (50–55). The synergistic potential of the optical and nuclear modalities has been recognized as a promising platform that allows the quantitative assessment of fluorescence-based imaging. The complementary nature arises from the shared and overlapping characteristics, namely the use
of labeled probes, photon processing, comparable detection sensitivities (high picomolar-femtomolar range), and the need to extract quantitative data. (43).

Dual labeling provides a framework that leverages the key strengths of each technique and overcomes the individual drawbacks from fluorescence-based and radionuclide-based monolabeled probes. More precisely, the development of such dynamic platforms allows the integration of signal reporters that synergistically provide higher spatiotemporal resolution and higher penetration depth, by means of low energy (1-2 eV) and high-energy photons (~80-511 keV), respectively. Dual labeling also provides intrinsic validation through correlation of co-localized NIRF/radionuclide signals in tissues. Moreover, dual labeling serves as a method to quantitatively assess pre-clinical NIRF imaging using standard nuclear imaging descriptors such as % injected dose per gram of tissue (%ID/g) (50–55). Importantly, dual labeling has the potential to extend preoperative surgical planning into the OR for real-time intraoperative cancer detection.

Given the ambitious nature of the dual labeling strategy, several design challenges need to be addressed. Depending on the structural scheme, the final multimodal reporter typically consists of a ligand, a linker, a chelator and the fluorescent dye – components which taken all together may be as large as the targeting moiety itself (50, 51, 55). In designing a NIRF/nuclear probe, maximum retention of optical characteristics, radiochemical properties, receptor affinity and biodistribution is desirable. Thus, careful consideration of the type/position of labels and the size relation is critical. Indeed, the literature is replete with instances where the chelator-isotope combination, radiochemical formulation or increase in molecular weight, significantly influenced the dye stability (56), coordination chemistry (57) or pharmacodynamic/pharmacokinetic properties (58–60), respectively, via alterations in the charge distribution and hydrophobicity characteristics.

Heptamethine cyanine dyes are the most commonly used fluorophores for dual labeling based on their longer-wavelength fluorescence, high quantum yield, high photostability and a range of bioconjugatable derivatives (61, 62). This class of NIR dyes consists of aromatic
heterocycles bridged by polymethine chains, which may be functionalized with cyclohexenyl substitutions to improve their photophysical properties. Additionally, sulfonic acid groups have been added to certain dyes to increase hydrophilicity. Nonetheless, the inherent lipophilic nature of fluorescent molecules, coupled with their generally uneven charge distribution, pose challenges in maintaining the rapid clearance properties of peptides. Specifically, IR800 has a hydrophobic core with a highly anionic surface charge and has been reported to primarily clear through the liver (63, 64). Previously, it has been shown that upon IR800 conjugation, a cyclic Arg-Gly-Asp (RGD) analog had a -4 net charge with an unbalanced charge-to-hydrophobicity distribution. Although the tumor targeting capabilities were not affected by such physicochemical properties, the IR800 conjugated RGD analog showed higher nonspecific accumulation in the liver and abdominal/thoracic walls when compared to a fluorescent RGD analog with 0 net charge and a well-balanced charge distribution (64).

Proof-of-concept studies have shown that dual labeling can be practical in the pre-clinical setting for the characterization of promising candidates for targeted FGS. For instance, the RGD peptide has been functionalized with the ICG analog, cypate, and 111In-DOTA for angiogenesis evaluation via αvβ3 integrin overexpression (65). The multimodal RGD derivative was found to retain in vitro receptor affinity and to target tumors in vivo as determined by consistent radioactive and fluorescence intensity data, indicating labeling did not interfere with binding properties. In another study, Zhang et al. developed the first bimodal positron emission tomography (PET)/fluorescent probe for gastrin-releasing peptide receptor targeting and assessed receptor-binding affinity upon dye conjugation (66). Using PET/CT and fluorescence imaging along cross-validation via biodistribution studies, the authors showed receptor-mediated uptake and high multimodal image contrast with potential for clinical translation. Similarly, Baranski et al. reported the synthesis and evaluation of a library of dual labeled agents derived from the radiotracer 68Ga-PSMA-11 for prostate-specific membrane antigen targeting (67). The authors found that the development of fluorescent-PSMA-11 analogs is feasible and concluded that these multimodal agents are promising candidates for pre-, intra- and postoperative imaging of prostate cancer.
Selection of neuroendocrine tumors as a disease model for FGS

The selection of a clinically established ligand-receptor complex as a model system would logically support the characterization of an FGS agent. This rationale is especially applicable to the somatostatin ligand-receptor system, a hallmark in neuroendocrine tumors (NETs). NETs are a heterogeneous family of slow-growing, poorly understood neoplasms with a mostly sporadic etiology and small familial risk (68) that would greatly benefit from the advantages provided by FGS given their propensity to form micrometastases (69–71). Although NETs are classified as relatively uncommon tumors, the 20-year limited-duration prevalence rate for the US population was estimated to have a 1.7-fold increase from 2004 (103,312) to 2014 (171,321) (72). They also have an annual overall incidence rate (6.3%) rising more rapidly than other more widespread cancers (i.e., lung and breast cancer) (73, 74). Because of the extended distribution of neuroendocrine cells across the body, NETs have a large spatial incidence encompassing the foregut, midgut and hindgut, with primary tumor sites occurring commonly in the lungs and gastrointestinal tract (68, 75). NETs are generally categorized as well-differentiated (low to intermediate grade) or poorly-differentiated (aggressive, high grade) based on factors such as histopathology, proliferation rate and functional status. Functioning tumors lead to additional debilitating symptoms due to excess peptide and hormone secretion and are routinely treated with somatostatin analogs (SSAs), the only proven therapy for hormonal hypersecretion in NETs (76).

The somatostatin receptor (SSTR) is a distinctive feature of NET biology and is overexpressed in 75-95% of the cases. Although the SSTR family is composed of 5 types of G-protein coupled transmembrane receptors, the SSTR type 2 (SSTR2) is the most abundant subtype and has proven to be a fundamental target for diagnostic imaging and treatment with SSAs (77). Two mechanisms of SSTR2 signaling and regulation are key for the clinical effectiveness of SSAs. First, SSTR2 coupling to adenylyl cyclase (AC) via pertussis-toxin sensitive G_{i/o} proteins inhibits AC, closes voltage-sensitive calcium channels and opens specific potassium channels (78). This in turn causes a reduction in two critical second messengers,
cyclic adenosine monophosphate (cAMP) and cytosolic calcium, which results in a synergistic inhibitory effect on hormonal hypersecretion (77). Second, rapid receptor phosphorylation leads to the desensitization and internalization of the ligand-receptor complex via clathrin-coated pits (78) that allows the intracellular accumulation of SSAs. Given the current clinical use of SSAs that have been iteratively optimized over the last 30 years, NETs are an ideal disease model for the development of an SSA-based FGS agent.

**Evolution of SSAs and therapeutic management of NETs**

The bioengineering of the first generation FDA-approved, eight amino acid SSAs octreotide (peptide sequence, -D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol)) and lanreotide (peptide sequence, -β-D-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH₂) has served as the basis for the treatment of functioning NETs (77). The therapeutic benefits of these SSAs are a result of their ability to inhibit the secretion by and growth of SSTR2-overexpressing tumors. Octreotide was first synthesized in 1982 (79) and entered clinical trials in 1984 (80). The synthesis of lanreotide was first reported in 1987 (81) with clinical studies beginning in 1989 (82). Both SSAs are synthetic agonists that have high SSTR2 affinity, lower SSTR3/SSTR5 affinity and no SSTR1/SSTR4 affinity (77). Importantly, these SSAs activate SSTR2 at similar nanomolar concentrations compared to the native counterparts, somatostatin-14 and somatostatin-28 (83). Because of their highly improved in vivo stability, octreotide and lanreotide have been further functionalized with radioactive elements and structural complexes capable of trapping isotopes for nuclear imaging. Octreotide was the first SSA to obtain clinical importance (84), which led to the development of a radiolabeled analog, iodine-123 labeled Tyr-3-octreotide (TOC), in 1989 for localizing tumors with a gamma camera (85). This initial success was followed by FDA-approval of ¹¹¹In-labeled DTPA-D-Phe¹-octreotide (OctreoScan) in 1994 (86), the first radiopharmaceutical that was routinely used in the clinic for planar/SPECT imaging and radio-guided surgery. The subsequent introduction of the metal chelator, DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) (87, 88), remarkably improved the stability of
radioconjugates and permitted the implementation of PET isotopes (i.e., ⁶⁸Ga, ⁶⁴Cu) for NET imaging and theranostic isotopes (i.e., ⁹⁰Y, ¹⁷⁷Lu) for peptide receptor radionuclide therapy (PRRT).

Advances in the management of NETs have translated into earlier, more precise diagnoses with ⁶⁸Ga-DOTA-TOC and ⁶⁸Ga-DOTA-TATE (structural analog with acidic C-terminus) as the PET imaging gold standards. Additionally, improved therapeutic options have emerged, including PRRT (89) and the approval of new targeted drugs (90). Despite these advances, surgery remains the only potential curative treatment for NETs. Importantly, surgery for NETs is indicated not only in localized cases, but also in advanced stages of the disease for debulking and palliating the abnormal tissue/hormonal burden. Given the relatively indolent and slow-growing nature of NETs, diagnosis is often delayed for many years, and at the time of presentation, 40-70% of patients have nodal or liver metastases (76). Although surgery extends the overall survival even in cases presenting metastatic disease, patients with NETs have a 5-year recurrence rate of more than 90%. This recurrence is primarily attributed to residual disease and incomplete removal of the cancerous metastatic lesions (69–71, 76, 91). Gamma probe-guided ultrasound (92) and radio-guided surgery (93) have been implemented in the OR to improve the detection of deep-seated and visually-challenging NET deposits. The added advantage of radio-guided surgical navigation via audible signal is that the SSTR2-targeting radiopharmaceutical used intraoperatively can also be used pre- and postoperatively for surgical planning and postoperative surveillance imaging. However, the inability to obtain visual information on tumor location significantly limits this approach. Consequently, fluorescent SSAs are candidates that can address the limitations of radio-guided surgery.

**The multimodality chelator (MMC) as a novel strategy for validating an FGS agent**

Numerous examples of targeted optical probes in pre-clinical stages for NET diagnosis and image-guided surgery are found in the literature (94). For instance, octreotide, octreotate, TOC and TATE (Tyr-3-octreotate) have been coupled to a range of visible and NIR dyes including
fluorescein (95), rhodamine derivatives (96) and cyanine dye derivatives (97–99). However, validated FGS agents for SSTR2 targeting are lacking.

To support the validation and translation of FGS agents, we propose the application of a novel, radioactive linker known as a multimodality chelator (MMC) that maximizes the distance between the pharmacophore and the fluorescent label (100). The implementation of the MMC permits “true” quantification and cross-validation through NIRF/nuclear signal co-localization. We apply a translational focus to our dual labeling strategy with the MMC by developing a fluorescent analog of the PET imaging gold standard in NETs, $^{68}$Ga-DOTA-TOC. Thus, **we hypothesize that SSTR2 targeting with $^{68}$Ga-DOTA-TOC would serve as a proven model for a dual-labeled SSA for FGS.**

To test this hypothesis, DOTA was substituted with the MMC, which minimized the steric effects of dye labeling, while allowing the retention of the chelator-peptide footprint of DOTA-TOC. The role of the MMC was subsequently redefined to serve as a radioactive linker that maximizes the distance between the pharmacophore (TOC) and the NIRF label (IR800). The arrangement of MMC(IR800)-TOC (Fig. 3) is possible because of the versatility afforded by the macrocyclic compound DO2A ($^{1,4,7,10}$-tetraazacyclododecane-1,7-bis(t-butyl acetate)), which structurally mimics DOTA, but permits selective functionalization of the chelator. Upon synthesis of an MMC with acetate and azide-containing pendant arms, Tyr$^3$-octreotide (TOC) conjugation was first performed using solid-phase peptide synthesis, and the resulting intermediate was then fluorescently labeled with IR800 via copper-free click chemistry in solution, respectively.
Figure 3. Structure and components of the SSTR2-targeted intraoperative imaging agent. MMC-mediated dual labeling enables quantitative characterization of the fluorescent somatostatin analog, MMC(IR800)-TOC.
Previous efforts to develop somatostatin analogs for nuclear/NIRF imaging have been attempted and major limitations were identified. For instance, Santini et al. successfully synthesized the fluorescent/radiolabeled hybrid Cy5-\textsuperscript{111}In-DTPA-Tyr\textsuperscript{3}-octreotate (101); however, upon dye conjugation, loss of binding properties was observed. In vitro uptake studies showed a 27.6-fold decrease in receptor binding compared to the monolabeled tracer \textsuperscript{111}In-DTPA-Tyr\textsuperscript{3}-octreotate (33.76\%±1.22\% applied dose vs. 1.32\%±0.02\%). In another study, Edwards et al. developed the multimodal SSA, cypate-labeled \textsuperscript{64}Cu/\textsuperscript{177}Lu-DTPA-Y3-TATE (102). Results showed that despite having high in vitro binding affinity, the multimodal agent failed to internalize. This indicates that the addition of cypate altered the mechanism of action of the somatostatin agonist Y3-TATE. It was also reported that the high in vitro affinity did not translate to in vivo tumor binding (≤1\% ID/g). Additionally, the probe had predominant hepatobiliary clearance (>90\% ID/g of liver) and low kidney clearance, which is the inverse of traditional peptide-based radiotracers. The authors attributed the low in vivo accumulation to the loss of internalization rate, a hallmark of successful SSTR2-agonist imaging agents.

Our approach to dual labeling yielded the first bioactive fluorescent analog of a clinical PET radiotracer (100), a strategy that further supports validation and translational efforts by integrating components that can address current scientific concerns and regulatory hurdles. Using \textsuperscript{68}Ga-DOTA-TOC provides a foundation that aligns with current clinical practices in NET management and a benchmark to guide optimization strategies. The use of the MMC technology potentially extends the pre- and postoperative PET utility of \textsuperscript{68}Ga-DOTA-TOC into the surgical suite, while generating a method to quantitatively validate the resulting intraoperative imaging agent. Importantly, \textsuperscript{68}Ga-DOTA-TOC would serve as an accompanying diagnostic for identifying candidates that would benefit from FGS with Ga-MMC(IR800)-TOC. In this project, we assess the performance and potential clinical utility of Ga-MMC(IR800)-TOC with the following specific aims:

**Aim 1. To determine the optimal radiosynthesis for \textsuperscript{68}Ga-MMC(IR800)-TOC:** using clinically
relevant radiochemistry formulations (NaCl/acetone/fractionation), conditions will be optimized based on high labeling efficiency and maximum retention of fluorescence intensity after labeling.

a) To quantify the radiochemical yield: radio-HPLC will be used to determine crude $^{68}$Ga complexation.

b) To assess the optical integrity: a fluorescence reader will be used to assess dye intensity after radiosynthesis and normalized to unprocessed controls.

Aim 2. To characterize the binding properties of $^{68}$Ga-MMC(IR800)-TOC in vitro: quantitation of receptor binding properties with radioligand assays will allow observation of SSTR2-cellular uptake and specificity.

a) To quantify SSTR2-binding: cellular uptake will be examined using HCT116 cells that stably overexpress SSTR2 (HCT116-SSTR2) and compared to $^{68}$Ga-DOTA-TOC.

b) To determine the specificity for SSTR2-binding: blocking studies will be performed with increasing amounts of the SSTR2-competitor, octreotide.

Aim 3. To assess the imaging properties of $^{68}$nat$^{68}$Ga-MMC(IR800)-TOC in xenografts: using an animal model with subcutaneous HCT116-SSTR2 tumors, the ability of the agent to provide tumor contrast will be assessed in vivo and validated ex vivo.

a) To identify differences in the pharmacokinetics of $^{68}$Ga-MMC(IR800)-TOC and $^{68}$Ga-DOTA-TOC: effects of dye labeling on the radiotracer will be determined by tissue biodistribution studies.

b) To determine specificity for localizing SSTR2-overexpressing xenografts: using PET/CT and NIRF imaging, in vivo specificity (competition, non-targeted, control cell line) for cancer tissue will be assessed and validated.
CHAPTER 2: MATERIALS AND METHODS
General methods

All chemicals were purchased from Sigma-Aldrich unless otherwise noted. Reversed-phase high-performance liquid chromatography (HPLC) was performed on an analytical Hitachi LaChrom system using a Kinetex C18 column (2.6 μm) (Phenomenex) with a mobile phase of A = 0.1% TFA in H₂O, B = 0.1% TFA in CH₃CN (gradient: 0 min, 10% B; 12 min, 90% B); flow rate, 1 mL/min. Radiochemical purities of ≥95% were assessed by radio-HPLC using an in-line radioactive detector (Berthold Technologies). ¹H NMR and ¹³C NMR spectra were recorded at ambient temperature using 600 MHz IBM-Bruker Avance NMR spectrometers. Chemical shifts (δ) were reported (in ppm) downfield of tetramethylsilane.

Labeling of MMC(IR800)-TOC with ⁶⁸Ga, ⁶⁷Ga, and Ga

⁶⁸Ga radiolabeling was performed using the acetone, NaCl and fractionation methods. For the acetone method, radiolabeling was performed as previously described (100). Briefly, ⁶⁸Ga was eluted from a ⁶⁸Ge/⁶⁸Ga-generator with 5 mL of 0.1 M HCl and adsorbed onto a Strata-X cation exchange cartridge (Phenomenex). After purging residual HCl from the cartridge, ⁶⁸Ga was collected with 98% acetone/0.02 M HCl (v/v) and 1 mCi was added to 20 nmol of MMC(IR800)-TOC (MW: 2835.1 g/mol) in 0.2 M sodium acetate (pH 4). Samples were heated at 95° C for 15 min. Following Sep-Pak Light C18 (Waters) purification, the product was diluted with PBS and analyzed by radio-HPLC. For the NaCl method, the radioactive solution was processed identically to the acetone method with the exception of using a Bond Elut cation exchange cartridge (Agilent), a 5 M NaCl/5.5 M HCl solution as an eluent and 2 M sodium acetate (pH 4) as the reaction buffer. For the fractionation method, ⁶⁸Ga was eluted from the generator with 5 mL of 0.1 M HCl and collected separately as five 1 mL fractions. Only the fraction with the highest radioactivity was selected for radiolabeling. 100 μL of peak fraction containing 0.3-0.4 mCi of ⁶⁸Ga was then directly added to 20 nmol of MMC(IR800)-TOC in 2 M sodium acetate (pH 4). Finally, incubation, purification and analysis were performed identically to the acetone/NaCl methods.
For $^{67}$Ga radiolabeling, $^{67}$Ga-citrate was purchased from a radiopharmacy (Cardinal Health) and was added to an equal volume of 0.1 M HCl to produce $^{67}$GaCl$_3$. The radioactive solution was then processed identically to $^{68}$Ga using the acetone method, with the exception of using 0.5 M ammonium acetate (pH 4.5) as the reaction buffer. Cold Ga labeling was performed with non-radioactive gallium according to methods established above for the radiolabeled compounds. 60 nmol was mixed with a 4-fold molar excess of GaCl$_3$ and heated at 95°C for 15 min. The crude mixture was purified by ultrafiltration and the final product was characterized by HPLC. Ga-MMC(IR800)-TOC: MS, ESI+: m/z calculated for C$_{135}$H$_{170}$N$_{22}$O$_{34}$S$_2$Ga, 2905.01; found m/z, 969.1 (M+3H)$^{3+}$.

**Optical characterization upon radiolabeling**

MMC(IR800)-TOC was radiolabeled with $^{68}$Ga using the fractionation, acetone and NaCl methods as previously described with the exception of using 1 nmol for the reaction. Briefly, incubation was performed for 15 min at 95°C in eppendorf vials. Reaction mixtures were purified, collected in 1:1 saline ethanol solution and allowed to cool down for 30 minutes at room temperature. Samples were then transferred to black 96-well half-area plates with clear bottom (Greiner Bio-One). Fluorescence intensity was measured using an Odyssey plate reader (LI-COR) and normalized to unprocessed MMC(IR800)-TOC in 1:1 saline ethanol solution. For the mock, nonradioactive solutions, MMC(IR800)-TOC was processed identically to the radioactive solutions with the exception of not adding $^{68}$Ga. Each experiment was performed in triplicate.

**Cell culture and animal models**

Athymic female nu/nu mice (Charles River Laboratories) were housed under standards of the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Health Science Center at Houston and maintained on normal rodent chow. HCT116-SSTR2 cells were kindly provided by Dr. Carolyn J. Anderson (University of Pittsburgh, Pittsburgh, PA, USA).
HCT116 cells (CCL-247) were purchased from ATCC. HCT116-SSTR2 and HCT116-WT cell lines were cultured in RPMI 1640 medium with 10% (v/v) fetal bovine serum (FBS) and maintained at 37°C with 95% humidity and 5% CO₂ atmosphere. HCT116-SSTR2 cells were additionally supplemented with 100 µg/ml Zeocin (Gibco). For all procedures, mice were anesthetized with 1-2% isoflurane. For xenografting, 6-8 weeks old mice were subcutaneously injected with 1×10⁶ HCT116-SSTR2 or HCT116-WT cells in matrigel (Corning):PBS (1:1) in the shoulder. Studies were conducted 3-4 weeks post implantation when tumor size reached approximately 5-10 mm maximum diameter.

**Measurement of intracellular cAMP with the GloSensor assay**

HEK293-HA3-rsstr2-Glo cells, expressing both the GloSensor 22F cAMP plasmid (Promega) and HA-tagged SSTR2 (from Dr. A. Schonbrunn Lab, The University of Texas Health Science Center at Houston), were seeded at 100,000 cells/well in 96 well plates. After 24 h, the medium was aspirated and replaced with 90 µL of warm (28°C) equilibration medium (DMEM + 10% FBS + 10 mM HEPES + 2% D-Luciferin). Plates were pre-incubated in a dark humidified chamber at 28°C for 2 h and then placed in a PolarStar Optima multiplate reader (BMG Labtech). Basal bioluminescence was measured and then 10 µl of NKH477 (final concentration = 10 µM) was added either with or without the appropriate SSA concentration. Readings were taken every 2.5 min for 1 h. Data shown were obtained 20 min after agonist addition and are expressed as a percentage of the luminescence measured in the presence of NKH477 alone. Reprinted (adapted) with permission from Sukhen C. Ghosh, Servando Hernandez Vargas, Melissa Rodriguez, et al. Copyright (2017) American Chemical Society. doi: 10.1021/acsmedchemlett.7b00125. These experiments were performed by Dr. Melissa Rodriguez and Dr. Agnes Schonbrunn in the Department of Integrative Biology and Pharmacology, McGovern Medical School, The University of Texas Health Science Center at Houston (100).
Measurement of receptor internalization

Changes in cell surface expression of SSTR2 were measured in HEK293-HA3-rsstr2 cells (from Dr. A. Schonbrunn Lab, The University of Texas Health Science Center at Houston) using ELISA, as previously described (78). Briefly, HEK293-HA3-rsstr2 cells were incubated with labeled peptides for 30 min at 37°C. After washing, cells were fixed, blocked with 1% BSA for 30 min, and incubated overnight at 4°C with mouse anti-HA antibody (1:10,000). Cells were then washed with PBS and incubated at r.t. for 1 h with goat anti-mouse HRP-labeled secondary antibody (1:10,000). Cell surface receptor level was determined by incubating for 45-60 min with ABTS and then measuring optical density at 405 nm. Reprinted (adapted) with permission from Sukhen C. Ghosh, Servando Hernandez Vargas, Melissa Rodriguez, et al. Copyright (2017) American Chemical Society. doi: 10.1021/acsmedchemlett.7b00125. These experiments were performed by Dr. Melissa Rodriguez and Dr. Agnes Schonbrunn in the Department of Integrative Biology and Pharmacology, McGovern Medical School, The University of Texas Health Science Center at Houston (100).

Radioactive uptake studies

HCT116-SSTR2 cells were seeded in 96-well plates (200,000 cells/well) and incubated with a 10 nM solution of $^{68}$Ga-MMC(IR800)-TOC or $^{68}$Ga-DOTA-TOC at 37°C for 1 h. For blocking, a 10 and 100-fold excess of octreotide was added prior to addition of the radiotracers to determine receptor specificity. At the end of the incubation period, cells were pelleted, media was removed, and cells were washed three times with PBS. The cells were then collected and radioactivity was quantified in a Wizard® automated γ counter (Perkin Elmer) to determine uptake as percent of total radioactivity added. The procedure was repeated using non-SSTR2 expressing HCT116-WT cells. Non-targeted $^{68}$Ga-MMC(IR800) was also tested in both cell lines to further demonstrate specificity. Each experiment was performed in triplicate. Reprinted (adapted) with permission from Sukhen C. Ghosh, Servando Hernandez Vargas, Melissa Rodriguez, et al. Copyright (2017) American Chemical Society. doi: 10.1021/acsmedchem-
Biodistribution in healthy mice

Normal 4-6 week old female, athymic, nude mice were injected intravenously with 740 kBq (20 μCi) of $^{68}$Ga-MMC(IR800)-TOC or $^{68}$Ga-DOTA-TOC. Under anesthesia, the mice were euthanized by cervical dislocation at 15 min, 1, and 3 h post-injection. Selected tissues were excised, weighed, and counted for radioactivity using the $\gamma$ counter. The results were expressed as percentage of the injected dose per gram of tissue (%ID/g) and represent the mean±SD of $n = 3$ mice/time point. The total injected activity per mouse was determined from a known aliquot of the injected solutions.

**In vivo PET/CT imaging**

Mice ($n=3$) were intravenously injected with 5.55 MBq (150 μCi, 4 nmol) of $^{68}$Ga-MMC(IR800)-TOC and non-invasive imaging was performed at 1 and 3 h after injection on a Siemens Inveon small-animal PET/CT scanner as previously described (57). Region-of-interest analysis was performed with the vendor software package (Inveon Research Workplace) to obtain tumor-to-background (TBR) ratios.

**In vivo NIRF imaging**

Mice ($n = 5$) were intravenously injected with Ga-MMC(IR800)-TOC (2 nmol, 5.67 μg) and imaging was performed at 3 and 24 h post-injection. *In vivo* NIRF images were acquired for 200 ms without background subtraction using a custom-built electron-multiplying charge-coupled device (EMCCD) fluorescence imaging system at $\lambda_{ex}/\lambda_{em} = 785/830$ (103), and image analysis was performed with the ImageJ software package (NIH). At the conclusion of the imaging studies, the mice were euthanized and selected organs were excised and underwent *ex vivo* NIRF imaging using an IVIS Lumina II (Perkin Elmer). For the selection of a radiolabeling method for
in vivo applications, mice (n = 4 per group) were imaged at 24 h post-injection as previously described with the exception of administering $^{68}$Ga-MMC(IR800)-TOC (0.5 nmol, 1.42 µg).

**Biodistribution and ex vivo imaging**

Mice with HCT116-SSTR2 tumors were intravenously injected with 370 kBq (10 µCi, 2 nmol) of $^{68}$Ga-MMC(IR800)-TOC or $^{67}$Ga-MMC(IR800)-TOC, and euthanized by cervical dislocation under anesthesia at 3 h and 24 h post-injection, respectively. Selected tissues were excised and underwent ex vivo optical imaging using an IVIS Lumina II with the following settings: lamp level (high), excitation (745 nm), emission (ICG), epi-illumination; binning (S); FOV (C, 10); f-stop (2); acquisition time (1 s). Region of interest analysis was performed with the vendor software package (Living Image) to measure fluorescence signals and obtain tumor-to-tissue ratios. Parameters were the same for all acquired images. At the completion of the optical imaging studies, tissues were weighed and counted for radioactivity using a Wizard$^2$ automated γ counter. The total injected activity per mouse was determined from an aliquot of the injected solutions. The results were expressed as %ID/g and represent the mean±SD of n = 5 mice/time point. To examine specificity, uptake in SSTR2 negative HCT116-WT xenografts was examined by in vivo and ex vivo optical imaging 24 h after injection of Ga-MMC(IR800)-TOC (2 nmol). Tumors and key organs (muscle, pancreas, small intestine) underwent cryoconservation by embedding in OCT and freezing on dry ice for subsequent mesoscopic and microscopic analysis. The non-targeted Ga-MMC(IR800) analog was evaluated identically to the targeted agent.

**Mesoscopic and subcellular localization of Ga-MMC(IR800)-TOC in tissue sections**

Frozen sections (10 µm) of muscle, pancreas, small intestine and tumors (HCT116-SSTR2 and HCT116-WT) from animals that were injected with Ga-MMC(IR800)-TOC (2 nmol) 24 h prior to necropsy were used to localize the IR800 signal within the tissue. Using an Odyssey slide scanner (LI-COR), the sections were scanned and the fluorescence intensities in the 800 nm channel were quantified on 16-bit images using ImageJ. ROIs were drawn around the outline
of each organ and means±SD were calculated using GraphPad Prism (n=3 mice/group). Adjacent sections underwent hematoxylin and eosin (H&E) staining to permit morphological analysis of the tissue.

To analyze Ga-MMC(IR800)-TOC distribution and receptor specificity at the cellular level, frozen sections of HCT116-SSTR2 and HCT116-WT tumors (24 h post 2 nmol Ga-MMC(IR800)-TOC injection) were fixed in 4% cold paraformaldehyde for 10 min and embedded in Mowiol mounting medium. For counterstaining, we added NucSpot Live 488 nuclear stain (Biotium) to the mounting medium (1:1000 dilution directly in Mowiol). Microscopic images were acquired on a confocal microscope (SP8, Leica), equipped with a 730 nm laser for IR800 detection and a 488 nm laser for NucSpot Live detection, in combination with appropriate filters. These experiments were performed by Dr. Susanne Kossatz and Dr. Thomas Reiner in the Department of Radiology, Memorial Sloan Kettering Cancer Center, New York.

**Immunohistochemical staining of SSTR2**

To detect SSTR2 expression, immunohistochemistry (IHC) was carried out on frozen sections of xenograft tumor tissue (HCT116-SSTR2, HCT116-WT) using the Discovery XT processor (Ventana Medical Systems, Tucson, AZ) at the Molecular Cytology Core Facility of Memorial Sloan Kettering Cancer Center. After thawing, sections were baked at 50ºC for 1 h, followed by a 30 min incubation with Background Buster solution (Innovex, Richmond, CA). Sections were then incubated with the anti-SSTR2 rabbit monoclonal antibody (Abcam) at 2.2 µg/ml for 5 h, followed by a 1 h incubation with biotinylated goat anti-rabbit IgG (Vector Labs). For detection, a DAB detection kit (Ventana Medical Systems) was used according to the manufacturer instructions. Sections were counterstained with H&E and cover-slipped with Permoun (Fisher Scientific). Slides were digitalized using a MIRAX Slide Scanner (3DHISTECH). These experiments were performed by Dr. Susanne Kossatz and Dr. Thomas Reiner in the Department of Radiology, Memorial Sloan Kettering Cancer Center, New York.
the support of the Radiochemistry & Molecular Imaging Probes Core Facility and Molecular Cytology Core Facility at Memorial Sloan Kettering Cancer Center.

**Statistical analysis**

Graphs and calculations were performed with GraphPad Prism (v 5.01). All data are presented as mean±standard deviation (SD) as indicated. Group comparisons were performed with one-way ANOVA (with Bonferroni’s post-tests) or two-tailed t-tests.
CHAPTER 3: RESULTS
MMC(IR800)-TOC efficiently chelates $^{68/67}_{\text{nat}}$Ga using clinically relevant formulations

DOTA provides high radiochemical yields alongside remarkable *in vivo* stability due to its kinetic inertness. Thus, by developing MMC(IR800)-TOC to structurally mimic the chelator-peptide footprint from DOTA-TOC, we hypothesized that our multifunctional chelator retains high labeling efficiency using clinically applied formulations.

$^{68}$Ga is a positron emitter ($\beta^+ = 89\%$, $E_{\text{avg}} = 740$ keV, $E_{\text{max}} = 1899$ keV) with desirable characteristics for peptide-based targeted imaging. With its 67.71 min half-life, $^{68}$Ga is compatible with the rapid pharmacokinetic profile of radiopeptides (104). Commercial $^{68}$Ge/$^{68}$Ga generators are available for on-site production of $^{68}$Ga. Thus, $^{68}$Ga availability is not necessarily dependent on a cyclotron schedule, but on the parent radionuclide in the generator ($^{68}$Ge $t_{1/2} = 270.95$ days) (105). By contrast, $^{67}$Ga is a gamma emitter ($t_{1/2} = 3.26$ days, $E_{\text{max}} = 394$ keV) that has been used in its salt form (i.e., $^{67}$Ga-citrate) as a radiopharmaceutical for assessing tumors, inflammation, infectious processes, etc., via scintigraphy and single-photon emission computed tomography (SPECT) imaging (106). However, $^{67}$Ga is produced by a cyclotron facility and distributed by radiopharmacies.

Our results show that $^{68}$Ga-MMC(IR800)-TOC production using the fractionation, acetone and NaCl formulations is achieved with high radiochemical yield (>80%) (Figs. 4a-c) and with >99% radiochemical purity following purification with a C-18 cartridge (Fig. 5). Figure 5 also shows the corresponding absorbance at 280 and 780 nm for purified $^{68}$Ga-MMC(IR800)-TOC. For $^{67}$Ga-MMC(IR800)-TOC radiosynthesis, a modified version of the Zhernosekov et al. formulation was developed in our laboratory as described in the methods section. The final $^{67}$Ga-product was produced with high radiochemical yield (72.7±5.1%, uncorrected for decay) and >99% radiochemical purity following purification (Figs. 6a and 6b). The stability was examined by incubating $^{68}$Ga-MMC(IR800)-TOC in mouse serum at 37°C for 3 h and analyzed by radio-HPLC (Fig. 7). No significant breakdown products or demetalation occurred following incubation. Finally, Ga-MMC(IR800)-TOC was produced using non-radioactive gallium and confirmed via mass spectrometry. These results indicate minimal impact of dye conjugation on the chelation
properties of the macrocycle. Moreover, they show that the MMC is a suitable DOTA substitute for Ga ions and can be used with multiple labeling methods.
Figure 4. Radiolabeling MMC(IR800)-TOC with clinically relevant $^{68}$Ga formulations. (a) Labeling conditions for each method. (b) HPLC chromatograms showing the radioactive trace of crude, non-decay corrected $^{68}$Ga-MMC(IR800)-TOC. (c) Radiochemical yields using fractionation, acetone and NaCl radiosynthetic methods. Data are presented as mean ± standard deviation (n=3).
Figure 5. Purified $^{68}$Ga-MMC(IR800)-TOC. HPLC chromatograms showing absorbance of purified $^{68}$Ga-MMC(IR800)-TOC at 280 and 780 nm, and the radioactive trace.

Figure 6. $^{67}$Ga-MMC(IR800)-TOC. HPLC chromatograms showing the radioactive trace of (a) crude and (b) purified $^{67}$Ga-MMC(IR800)-TOC.
Figure 7. Stability of $^{68}$Ga-MMC(IR800)-TOC in mouse serum. HPLC chromatograms showing the radioactive trace of $^{68}$Ga-MMC(IR800)-TOC in serum up to 3 h.
Comparison of normalized fluorescence upon subjection to radiolabeling

Given the differences in buffer concentrations and eluting solutions when utilizing the acetone, fractionation and NaCl methods for producing $^{68}$Ga-MMC(IR800)-TOC, we evaluated whether the selection of radiolabeling scheme resulted in differences in fluorescence intensity.

The normalized fluorescence intensity for each method is shown in Figure 8. For all 3 methods, the mock, nonradioactive solutions retained >94% of the initial fluorescence in NaOAc buffers ranging from 0.2-2 M with eluting solutions specific for each scheme. Upon $^{68}$Ga addition, the acetone and fractionation methods showed similar fluorescence retention of 95.7±2.5% and 94.6±2.1%, respectively. The NaCl scheme resulted in the normalized fluorescence decreasing to a final value of 85.1±2.3%, which was significantly different to the acetone and fractionation formulations ($P < 0.05$). This indicates that the addition of $^{68}$Ga to the NaCl reaction mixture is a contributing factor leading to a reduction in NIRF signal. Although we cannot draw definitive conclusions on the reasons leading to the significant decrease in fluorescence using the NaCl formulation, these results suggest that the selection of radiolabeling method may alter the spectral properties of $^{68}$Ga-MMC(IR800)-TOC.
Figure 8. Normalized fluorescence intensity of MMC(IR800)-TOC. Radioactive and nonradioactive (mock) solutions were prepared using three radiolabeling schemes (fractionation, acetone and NaCl) and normalized with unprocessed MMC(IR800)-TOC. *P < 0.05. Data are presented as mean ± standard deviation (n=3).
Ga-MMC(IR800)-TOC retains intact pharmacological properties

The selection of the well-characterized SSTR2 agonist $^{68}$Ga-DOTA-TOC as a system for the development of a fluorescent counterpart provides an ideal benchmark to assess the pharmacological impact of conjugating IR800 via the MMC linker.

Figures 9a and 9b show the potency of peptide conjugates for cAMP inhibition and receptor internalization in HEK293-SSTR2 expressing cells. Using Ga-DOTA-TOC as a reference standard, we found that Ga-MMC(IR800)-TOC is able to maximally inhibit NKH477-stimulated cAMP formation with high efficacy in the sub-nanomolar range (0.066±0.012 nM). NKH477 is a water-soluble forskolin derivative that directly activates AC and catalyzes cAMP production (107). Also, Ga-MMC(IR800)-TOC effectively stimulates receptor internalization with an EC$_{50}$ of 48.7±9.9 nM, which was comparable to the EC$_{50}$ for Ga-DOTA-TOC (16.6±3.7 nM).

These studies indicate that the MMC scaffold successfully minimizes the effects of dye conjugation and allows the retention of agonist properties despite the addition of a bulky dye. Thus, the MMC is an effective scaffold for production of a fluorescent DOTA-TOC analog with intact pharmacological properties.

$^{68}$Ga-MMC(IR800)-TOC binds specifically to SSTR2 and comparably to $^{68}$Ga-DOTA-TOC

To further assess cellular binding, uptake and blocking of the radiolabeled hybrid SSA was investigated according to published protocols (58). Results showed that $^{68}$Ga-MMC(IR800)-TOC uptake was 25.0±1.7% (% of total radioactivity added) in HCT116-SSTR2 cells at 1 h (Fig. 9c). Uptake in parental, non-SSTR2 expressing, HCT116 cells was 1.9±0.9%. The findings were in agreement with $^{68}$Ga-DOTA-TOC uptake (21.5 ±3.7%, Fig. 9c), further suggesting that IR800 conjugation did not impact SSTR2-mediated binding capabilities. To further evaluate whether the MMC-dye complex was contributing to any non-specific binding, a non-targeted MMC analog, $^{68}$Ga-MMC(IR800), was incubated with both SSTR2-expressing and non-expressing HCT116 cells. Figure 9c shows that at 1 h, there is negligible uptake of $^{68}$Ga-MMC(IR800) by either cell line. Blocking studies with increasing concentrations of octreotide yielded a dose-dependent
reduction of \(^{68}\text{Ga}-\text{MMC(IR800)}\)-TOC binding (Fig. 9d). The presence of a 10 and 100-fold octreotide excess resulted in a 78.5±9.6% and 93.7±1.6% reduction in \(^{68}\text{Ga}-\text{MMC(IR800)}\)-TOC binding, respectively. Taken together, these results support the data from the \textit{in vitro} pharmacological assays and provide robust evidence that indicate receptor-mediated uptake of the dual-labeled agent.
Figure 9. *In vitro* characterization of $^{68}$Ga-MMC(IR800)-TOC. Potency of peptide conjugates for (a) cAMP inhibition and (b) receptor internalization in HEK293-SSTR2 expressing cells. (c) Cellular uptake and (d) blocking of peptide conjugates in HCT116-SSTR2 and wild type HCT116 cells. *P < 0.0001. Experiments for (a) and (b) performed by Dr. Melissa Rodriguez and Dr. Agnes Schonbrunn in the Department of Integrative Biology and Pharmacology, McGovern Medical School, The University of Texas Health Science Center at Houston.

**68Ga-MMC(IR800)-TOC undergoes time-dependent elimination in vivo**

High biostability and hydrophilicity are desirable properties for a clinical imaging radiopeptide since they favor the renal-urinary mode of excretion over hepatobiliary excretion, thus enabling tumor visualization in the abdominal cavity (108, 109). 68Ga-DOTA-TOC has high in vivo kinetic inertness and hydrophilicity (log P = -2.9) (110), allowing it to rapidly clear from non-target tissues and provide a low noise floor to enhance tumor detection sensitivity.

To evaluate the impact of dye conjugation on the pharmacokinetics, we performed radioactive biodistribution studies at 15 min, 1 h and 3 h post-injection and results are summarized in Figure 10 and Table 3. 68Ga-MMC(IR800)-TOC undergoes time-dependent elimination from circulation and healthy tissues up to 3 h post-injection. Kidneys were identified as the primary clearance route, which suggests that the fluorescent counterpart, despite the addition of multiple aromatic groups, retains the hydrophilic character of the parent radiopeptide. IR800 conjugation did increase clearance through the reticuloendothelial system (RES) (liver and spleen) and resulted in longer circulation in blood, however, the radioactive fractions in these tissues decreased progressively with time and may provide suitable tumor contrast in the abdomen region at a delayed time point (i.e., > 3 h). At other sites of interest, such as the lungs, high 68Ga-MMC(IR800)-TOC signal was persistent up to 3 h, while the 68Ga-DOTA-TOC signal was significantly higher at the pancreas up to 1 h (P < 0.05). The radioactive signal decreased over time for both agents in the remaining tissues.
Figure 10. Radioactive biodistribution in healthy mice. Tissue uptake values for $^{68}$Ga-MMC(IR800)-TOC and $^{68}$Ga-DOTA-TOC.

Table 3. Biodistribution results (%ID/g) for $^{68}$Ga-MMC(IR800)-TOC and $^{68}$Ga-DOTA-TOC.

<table>
<thead>
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<th>$^{68}$Ga-MMC(IR800)-TOC</th>
<th>$^{68}$Ga-DOTA-TOC</th>
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<tbody>
<tr>
<td></td>
<td>15 min</td>
<td>1 h</td>
</tr>
<tr>
<td>Blood</td>
<td>3.12 ± 0.02</td>
<td>1.42 ± 0.08</td>
</tr>
<tr>
<td>Liver</td>
<td>4.50 ± 0.13</td>
<td>3.09 ± 0.16</td>
</tr>
<tr>
<td>Kidney</td>
<td>20.91 ± 3.21</td>
<td>17.04 ± 1.10</td>
</tr>
<tr>
<td>Spleen</td>
<td>9.73 ± 2.38</td>
<td>6.05 ± 1.23</td>
</tr>
<tr>
<td>Heart</td>
<td>2.80 ± 0.35</td>
<td>1.44 ± 0.03</td>
</tr>
<tr>
<td>Stomach</td>
<td>3.37 ± 0.33</td>
<td>2.53 ± 0.30</td>
</tr>
<tr>
<td>Intestine</td>
<td>2.64 ± 0.46</td>
<td>1.59 ± 0.66</td>
</tr>
<tr>
<td>Muscle</td>
<td>2.68 ± 1.42</td>
<td>0.90 ± 0.34</td>
</tr>
<tr>
<td>Bone</td>
<td>1.67 ± 0.11</td>
<td>1.53 ± 0.16</td>
</tr>
<tr>
<td>Pancreas</td>
<td>1.97 ± 0.04</td>
<td>1.05 ± 0.16</td>
</tr>
<tr>
<td>Lung</td>
<td>6.20 ± 1.45</td>
<td>5.23 ± 0.39</td>
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\^{68}\text{Ga-MMC(IR800)-TOC} \text{ binds to SSTR2 xenografts } \textit{in vivo} \text{ and is visualized using PET/CT}

\^{68}\text{Ga-DOTA-TOC} \text{ combines rapid tumor accumulation (80\% of the dose within 30 minutes), low non-specific binding and rapid renal clearance, with suitable contrast being achieved 1 to 1.5 h after agent administration (86). To examine the tumor targeting properties of the dual-labeled agent, MMC(IR800)-TOC was radiolabeled with } \^{68}\text{Ga and administered to mice bearing HCT116-SSTR2 xenografts for PET/CT imaging at 1 and 3 h post-injection. Accumulation at the tumor site can be seen at 1 and 3 h (Fig. 11a), with tumor-to-background ratios of 1.9±0.7 and 2.6±0.9 (Fig. 11b), respectively. PET/CT imaging was in agreement with the pharmacokinetic data obtained in healthy mice. The highest signal was observed in the kidneys, indicating renal clearance. Prominent lung and liver signal was also visualized at both time points, indicating involvement of the RES organs during clearance. These results provide evidence of } \textit{in vivo} \text{ SSTR2 targeting capabilities and suggest that delayed imaging (>> 3 h) may provide increased contrast in the thoracic and gastrointestinal regions.}
Figure 11. PET/CT imaging of HCT116-SSTR2 xenografts 1 and 3 h after injection of $^{68}$Ga-MMC(IR800)-TOC. (a) Coronal images and maximum-intensity projections (MIPs) are shown (arrow indicates tumor). (b) Ratio of the signal in tumor and background tissue. Data are presented as mean ± standard deviation (n=3).
Determination of optimal imaging time point

We previously observed that $^{68}$Ga-MMC(IR800)-TOC undergoes time-dependent clearance from circulation and tissues up to 3 h post-injection. Therefore, we selected the 3 h time point for the initial in vivo NIRF imaging experiments. The in vivo kinetics of non-radioactive Ga-MMC(IR800)-TOC were qualitatively and semi-qualitatively evaluated in mice bearing subcutaneous HCT116-SSTR2 xenografts using a custom-built EMCCD fluorescence imaging system.

Early optical imaging showed inconsistent tumor delineation, alongside prominent kidney signal and background fluorescence in the thoracic and abdominal walls (Fig. 12a). We hypothesized that this inconsistency was a result of low agent accumulation, slow elimination from background tissues or a combination of the two. Thus, NIRF imaging was repeated 24 h post-injection (Fig. 12a). Delayed imaging showed clear tumor delineation and contrast enhancement. Semi-qualitative analysis of the normalized fluorescent signal indicates that the contrast gain in the tumor is a combination of signal retention and a 2-fold signal reduction from background regions (Fig. 12b).
Figure 12. *In vivo* NIRF imaging of Ga-MMC(IR800)-TOC in mice. (a) Representative NIRF images of Ga-MMC(IR800)-TOC were acquired in HCT116-SSTR2 xenografts at early and delayed time points using a custom EMCCD fluorescence imaging system. Solid arrows indicate tumor, dashed arrows indicate kidney. (b) Normalized fluorescence signal in tumor and background tissue. * P< 0.005. Data are presented as mean ± standard deviation (n=5).
**Ex vivo quantitative analysis and nuclear/NIRF contrast determination**

A major advantage for dual labeling an FGS agent is the inherent ability to quantify its biodistribution for cross-validation of the optical data. Accordingly, the radioactive utility of the MMC was used to produce dual labeled conjugates for early (3 h, $^{68}$Ga) and delayed (24 h, $^{67}$Ga) biodistribution studies. *Ex vivo* evaluation by IVIS imaging of resected organs was in agreement with *in vivo* results (Fig. 13a). Agent uptake in the tumor was similar at 3 and 24 h and clearance was primarily through the kidneys. Moderate liver signal was observed at both time points, but the early lung fluorescence was reduced to background levels at 24 h indicating washout from non-target sites. Fluorescence was minimal at both time points in other healthy tissues and importantly in sites that endogenously express low/intermediate SSTR2 levels (small intestine, pancreas, and stomach) (111).

Analysis of fluorescence intensity is summarized in Figure 13b and Table 4, with corresponding optical contrast ratios shown in Figure 13c. Accumulation of the fluorescent signal in the tumor was rapid and sustained with no significant change up to 24 h, and was higher than non-clearance organs. Clearance was primarily through the kidneys, which showed constant signal over time. The NIRF signal decreased significantly at 24 h for most of the remaining tissues and remarkably in the small intestine (22.8%, $P < 0.01$), muscle (29%, $P < 0.05$) and lung (51.3%, $P < 0.01$). The reduction of the NIRF signal translated into 1.3 and 1.8-fold contrast enhancements in tumor-to-muscle ($P < 0.05$) and tumor-to-lung ($P < 0.05$) ratios, respectively. Importantly, the pancreas and small intestine had constant contrast ratios of >2.5, while for the lungs, the ratio increased from 1.40±0.40 to 2.52±0.27, which is critical since a TBR of at least 2 is generally accepted as being suitable for tumor delineation in the operating room.

Radioactive biodistribution results are summarized in Figure 13d and Table 5, with corresponding tumor-to-tissue ratios shown in Figure 13e. At 3 h, administration of $^{68}$Ga-MMC(IR800)-TOC resulted in high renal clearance (45.6±3.8 %ID/g), along with prominent accumulation in the lungs (6.7±0.9 %ID/g), liver (5.0±0.9 %ID/g), and stomach (4.5±0.8 %ID/g). Tracer accumulation of 3.54±0.85 %ID/g was measured in the tumor and was higher than...
pancreas (1.7±0.2 %ID/g) and small intestine (1.3±0.1 %ID/g). Analysis of the 24 h biodistribution data showed similar tumor uptake of $^{67}$Ga-MMC(IR800)-TOC compared to the 3 h group (4.26±1.08 %ID/g, $P > 0.05$). Importantly, reduction in signal was seen in most tissues that were not involved in clearance. Most notably, tracer signal decreased by 64.6% in muscle ($P < 0.01$) and 84.3% in blood ($P < 0.001$), yielding tumor-to-muscle and tumor-to-blood values of 18.9±3.7 and 11.5±3.4, respectively, and a 3.1-fold (muscle) and 7.1-fold (blood) increase from values obtained at 3 h. In tissues relevant to NET surgeries, tumor-to-tissue ratios improved significantly for the lung ($P < 0.01$) to a final value of 1.50±0.43 (2.8-fold increase).
Figure 13. Quantitative biodistribution assessment by dual labeling. (a) Ex vivo optical images (IVIS) of organs resected from HCT116-SSTR2 xenografts that were injected with $^{68}$Ga-MMC(IR800)-TOC or $^{67}$Ga-MMC(IR800)-TOC. Determination of (b) tissue fluorescence by analysis of IVIS imaging and (c) corresponding contrast ratios at major sites of NET incidence (pancreas, small intestine, lung) and selected non-target sites (muscle and blood). Determination of (d) radioactive uptake by gamma counting and (e) corresponding gamma counting ratios. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Data are presented as mean ± standard deviation (n=5). Average radiant efficiency displayed as ([p/s/cm²/sr]/[µW/cm²]). S.I., small intestine.
Table 4. Average radiant efficiency × 10³ ([p/s/cm²/sr]/[µW/cm²]) results for $^{68}$Ga-MMC(IR800)-TOC and $^{67}$Ga-MMC(IR800)-TOC.

<table>
<thead>
<tr>
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<th>$^{68}$Ga-MMC(IR800)-TOC</th>
<th>$^{67}$Ga-MMC(IR800)-TOC</th>
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<tbody>
<tr>
<td></td>
<td>3 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Tumor</td>
<td>231,420 ± 60,115</td>
<td>205,075 ± 22,691</td>
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<tr>
<td>Muscle</td>
<td>50,478 ± 9,203</td>
<td>35,825 ± 1,259</td>
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<td>Pancreas</td>
<td>90,390 ± 9,067</td>
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<td>S. Intestine</td>
<td>54,856 ± 4,742</td>
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<td>169,480 ± 38,156</td>
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<td>Liver</td>
<td>139,440 ± 17,199</td>
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<td>Spleen</td>
<td>58,998 ± 9,312</td>
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<td>Stomach</td>
<td>84,198 ± 11,288</td>
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<td>Kidney</td>
<td>1,152,800 ± 129,635</td>
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<td>Bone</td>
<td>87,696 ± 9,263</td>
<td>61,505 ± 7,635</td>
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<tr>
<td>Heart</td>
<td>60,228 ± 4,068</td>
<td>45,813 ± 12,630</td>
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<tr>
<td>Adrenal</td>
<td>93,834 ± 20,315</td>
<td>86,138 ± 42,056</td>
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Table 5. Biodistribution results (%ID/g) for $^{68}$Ga-MMC(IR800)-TOC and $^{67}$Ga-MMC(IR800)-TOC.

<table>
<thead>
<tr>
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<th>$^{68}$Ga-MMC(IR800)-TOC</th>
<th>$^{67}$Ga-MMC(IR800)-TOC</th>
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<tr>
<td></td>
<td>3 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Tumor</td>
<td>3.54 ± 0.85</td>
<td>4.26 ± 1.08</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.64 ± 0.12</td>
<td>0.22 ± 0.05</td>
</tr>
<tr>
<td>Pancreas</td>
<td>1.67 ± 0.21</td>
<td>1.44 ± 0.13</td>
</tr>
<tr>
<td>S. Intestine</td>
<td>1.29 ± 0.13</td>
<td>1.05 ± 0.21</td>
</tr>
<tr>
<td>Lung</td>
<td>6.68 ± 0.89</td>
<td>2.90 ± 0.83</td>
</tr>
<tr>
<td>Liver</td>
<td>5.04 ± 0.88</td>
<td>5.03 ± 0.54</td>
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<td>Spleen</td>
<td>3.16 ± 0.94</td>
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<td>Stomach</td>
<td>4.48 ± 0.77</td>
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<tr>
<td>Brain</td>
<td>0.12 ± 0.06</td>
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<td>Blood</td>
<td>2.38 ± 0.23</td>
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<td>Kidney</td>
<td>45.58 ± 3.77</td>
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<td>Adrenal</td>
<td>2.30 ± 2.85</td>
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</tr>
<tr>
<td>Urine</td>
<td>56.13 ± 18.23</td>
<td>5.14 ± 0.98</td>
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Specificity for SSTR2 imaging *in vivo*

SSTR2-mediated-targeting of our optical probe was evaluated by using 1) mice with HCT116-SSTR2 and HCT116-WT tumors, and 2) the non-targeted MMC analog, Ga-MMC(IR800), in HCT116-SSTR2 mice. *In vivo* imaging showed clear Ga-MMC(IR800)-TOC signal in HCT116-SSTR2 tumors but not in WT tumors lacking SSTR2 expression (Fig. 14a). *Ex vivo* IVIS imaging was in agreement with *in vivo* results (Fig. 14b) and subsequent image analysis showed a higher (*P* < 0.01) average radiant efficiency emitted from the HCT116-SSTR2 xenograft compared to the WT tumors and non-targeted analog (Fig. 14c). This, in turn, resulted in TBRs that were 1.8, 1.8, 2.3, and 2.4-fold higher than WT in the muscle, pancreas, small intestine, and lung of HCT116-SSTR2 mice, respectively (Fig. 14d).

Mesoscopic analysis of frozen sections confirmed the *in vivo* and *ex vivo* imaging results and showed specific, localized Ga-MMC(IR800)-TOC uptake only in HCT116-SSTR2 tumors (Fig. 15a). H&E staining showed that agent uptake was confined to viable areas of the HCT116-SSTR2 tumor, while no accumulation was observed in the WT counterpart (Fig. 15a). The signal in HCT116-SSTR2 tumors was 8.6-fold higher than in SSTR2-deficient tumors, while the signal in pancreas, muscle and small intestine are comparably low between the groups (Fig. 15b). On the microscopic level, we observed an intracellular agent accumulation in HCT116-SSTR2 tumors, while we did not detect the NIRF signal in HCT116-WT tumors (Fig. 15c).
Figure 14. *In vivo* and *ex vivo* specificity of Ga-MMC(IR800)-TOC. (a) *In vivo* NIRF imaging in HCT116-SSTR2 and HCT116-WT subcutaneous xenografts acquired 24 h post-injection of Ga-MMC(IR800)-TOC with a custom-built EMCCD fluorescence imaging system. The non-targeted Ga-MMC(IR800) in HCT116-SSTR2 mice was similarly evaluated. Arrows indicate tumor. (b) *Ex vivo* NIRF imaging of selected organs using an IVIS Lumina II. (c) Tissue fluorescence determined from analysis of IVIS imaging. (d) Optical contrast provided by the ratio of the average fluorescent signal in the tumor to sites of NET formation. * P< 0.05, ** P< 0.01, ***P< 0.001. Data are presented as mean ± standard deviation (n=5). Average radiant efficiency displayed as ([p/s/cm²/sr]/[µW/cm²]).
Figure 15. Multiscale imaging of Ga-MMC(IR800)-TOC uptake. (a) Confirmation of in vivo accumulation of Ga-MMC(IR800)-TOC in HCT116-SSTR2 and HCT116-WT xenografts and organs via frozen section imaging using the Odyssey NIR scanner. H&E sections provide morphological reference. (b) Quantification of the fluorescence signal of tumor and organ frozen section Odyssey scans (Means and standard deviations of n=3 animals/group). (c) Microscopic detection of in vivo injected Ga-MMC(IR800)-TOC. Frozen sections from resected HCT116-SSTR2 and HCT116-WT tumors were counterstained with a nuclear stain (Nucspot488), fixed and examined under a confocal microscope. An NIR signal was only detected in HCT116-SSTR2 tumor, corresponding to the SSTR2 expression in this model, while it was absent in SSTR2 negative HCT116-WT tumors. Experiments were performed by Dr. Susanne Kossatz and Dr. Thomas Reiner in the Department of Radiology, Memorial Sloan Kettering Cancer Center, New York.
Effect of injected dose on image contrast

The detection of small lesions while maintaining a high tumor-to-background ratio is a major challenge for FGS. The answer to the question of “what is the smallest amount of cancer that this technique can detect?” is a function of the sensitivity and specificity of both the optical agent and the imaging device (41). Thus, we evaluated the interplay between injected amount and contrast by comparing the effects of low (0.5 nmol) and high (2 nmol) dose administration of Ga-MMC(IR800)-TOC in SSTR2-expressing tumors at 24 h. Ex vivo IVIS imaging showed higher tumor fluorescence with the 2 nmol group. However, the higher dose also resulted in the amplified non-specific accumulation of signal in non-target and healthy tissues (Fig. 16a). Image analysis confirmed that although a larger dose yields a 3.8-fold increase ($P < 0.001$) in total fluorescent output in the tumor, it also elevates fluorescence in non-tumor tissues and raises the background signal by >6-fold in the pancreas, small intestine and lung ($P < 0.001$) (Fig. 16b). The increase in NIRF signal at 2 nmol/mouse resulted in an approximately 40% reduction in tissue contrast compared to 0.5 nmol/mouse (Fig. 16c). Since an elevated noise floor could offset the high signal in the tumor and impair intraoperative visualization of NETs, we identified 0.5 nmol as the starting point for future in vivo studies.
Figure 16. Effect of injection mass on optical contrast. (a) Macroscopic NIRF imaging of resected organs following administration of 0.5 nmol and 2 nmol. (b) Tissue fluorescence measured by IVIS imaging following injection of Ga-MMC(IR800)-TOC at 0.5 nmol and 2 nmol per mouse. (c) Optical contrast provided by the ratio of the average fluorescent signal in the tumor to sites of tumor formation. * P<0.05, ** P<0.01, ***P<0.001. Data are presented as mean ± standard deviation (n=5). Average radiant efficiency displayed as ([p/s/cm²/sr]/[µW/cm²]).
CHAPTER 4: DISCUSSION
The gap between pre- and intraoperative imaging emphasizes the tremendous difficulties of navigating the translational pathway and the need for new methods to validate FGS agents. In an effort to overcome these difficulties, we hypothesize that the clinical radiotracer $^{68}\text{Ga}$-DOTA-TOC would serve as an ideal system for developing a fluorescent analog for the specific targeting of SSTR2 for FGS in NETs. With the implementation of the innovative MMC linker, which functions as a spacer and radioactive core to bridge TOC and IR800, we were able to generate a fluorescent DOTA-TOC analog with minimal structural deviations. The application of the dual labeling strategy permits cross-validation through “true” quantification and NIRF/nuclear signal co-localization, while having $^{68}\text{Ga}$-DOTA-TOC as a clinical benchmark facilitates pre-clinical evaluation. Importantly, our approach complements SSTR2-targeted radiotracers (i.e., $^{68}\text{Ga}$-DOTA-TOC and $^{68}\text{Ga}$-DOTA-TATE). The pre-operative PET with a gold standard will accurately identify the number of lesions and will be combined with CT/MRI to establish a surgical plan. The surgeon can then navigate per standard of care to the appropriate location and utilize the contrast provided by Ga-MMC(IR800)-TOC to remove lesions.

Numerous methods are available for $^{68}\text{Ga}$-DOTA-TOC radiosynthesis. For testing $^{68}\text{Ga}$-MMC(IR800)-TOC labeling, we selected the fractionation, acetone and NaCl methods as formulations with translational relevance. Each one of these methods has advantages and disadvantages that define their suitability for specific applications. Fractionation is a well-established radiochemical method where $^{68}\text{Ga}$ is eluted from the $^{68}\text{Ge}/^{68}\text{Ga}$-generator in fractions and the portion with the highest activity (~80% of activity concentrated in 1 mL of eluate) is selected for direct incubation with the tracer (112). Concerns with $^{68}\text{Ge}$ and metal (i.e., Zn(II)) breakthrough drove the development of alternate radiosynthetic procedures that include pre-concentration and purification of the initial $^{68}\text{Ga}$ eluate using exchange chromatography. Zhernosekov et al. reported the now widely adopted successful purification of the $^{68}\text{Ga}$ eluate using a cation-exchanger with acetone recovery for high radiochemical yield (113). Recently, Mueller et al. has substituted the use of the organic solvent acetone (desirable for medical applications), with a NaCl-based solution for the production of radioconjugates with high specific
activities (114). Here, using these clinical radiochemistry formulations, we found that the MMC complex coordinates $^{68}$Ga, $^{67}$Ga and Ga with high radiochemical yield and stability in serum, which suggests that the MMC is a suitable DOTA substitute for in vivo applications and a versatile chelator for metals and radiometals. As we move forward with testing new dyes and targeting-peptides, this radiochemical flexibility would permit the customization and optimization of radiolabeling conditions.

In a dual labeled format, the performance of the fluorescent dye critically depends on the preservation of the stability and optical properties upon subjection to radiolabeling. Labeling formulations require a wide range of buffers, eluting solutions, pH ranges, heating temperatures, etc. These differing factors coupled to varying concentrations of radioactivity may impact the spectral properties of the dye. In a previous study by our group (51), it was shown that the relative brightness (RB) of IR800 remains relatively unchanged at room temperature in the presence of Ga and in NaOAc solutions ranging from 0.1-1.25 M and pH 4-6. In a follow up experiment, IR800 was added to 1.25 M NaOAc (pH 4) and heated to 95° C for 10 min with varying levels of $^{68}$Ga activity to simulate in vitro (0.6 mCi, low), in vivo (1.6 mCi, medium) and human studies (4.1 mCi, high). Results showed that while the RB remained >2 and similar to that of the non-radioactive buffer system for the low and medium activity doses, the radioactive solution with the highest dose caused the RB to drop below 2. Similarly, in the present study we evaluated whether the selection of radiolabeling scheme resulted in differences in fluorescence intensity. The fractionation and acetone formulations yielded the highest signal retention and results were in agreement with previous work by our group. The acetone formulation was selected for subsequent in vivo applications based on high fluorescence retention and the ability to purify/pre-concentrate $^{68}$Ga for production of $^{68}$Ga-MMC(IR800)-TOC with high specific activity.

Retention of pharmacological properties is another crucial pre-clinical endpoint when developing a targeted agent that combines NIRF and nuclear contrasts. Agonist-induced mechanisms have been a paradigm used by radiolabeled SSAs for nuclear imaging and PRRT. This paradigm is established on the putative ability of somatostatin agonists to inhibit cAMP
production and to induce the internalization of the ligand-receptor complex (77, 78). Previously, dual labeled SSAs have been reported to lose the ability to bind the receptor (101) and internalize (102) upon dye attachment. Thus, we investigated the impact of fluorescent functionalization on these fundamental radiotracer characteristics. We examined receptor kinetics with pharmacological assays and radioactive uptake studies, which indicated that $^{68}$Ga-MMC(IR800)-TOC maintains intact binding and molecular mechanisms of action at comparable $^{68}$Ga-DOTA-TOC potencies. These in vitro pharmacodynamic results have been confirmed by analysis of the subcellular localization of the agent using confocal microscopy (100).

Image contrast is dependent on agent accumulation in tumors and clearance from background tissues. In healthy mice, we found that $^{68}$Ga-MMC(IR800)-TOC underwent time-dependent elimination from circulation and background tissues up to 3 h post-injection. Since similar findings were shown by PET/CT imaging in SSTR2-overexpressing xenografts, we selected 3 h as the initial time point for in vivo NIRF imaging but found that tumors were not clearly visualized. Given the continuous washout of agent from normal tissues, we extended the imaging study to 24 h to enable further clearance and showed clear delineation of tumors in all mice. Since optical imaging is not quantitative, we produced radioactive analogs for early ($^{68}$Ga) and delayed ($^{67}$Ga) biodistribution studies to further evaluate tissue uptake. From those experiments, we confirmed that clearance from background organs was the major factor contributing to the improved focal signal being detected from the cancer lesion at 24 h. Importantly, tissue uptake and contrast ratios obtained by gamma counting were essentially identical to the ex vivo optical data and demonstrated the intrinsic utility of dual labeling as a tool for validating an FGS agent.

The efficacy of an FGS agent has been defined by FDA guidance documents as the ability to distinguish between normal and abnormal anatomy (42). Attempting to detect microscopic disease in a macroscopic setting, namely the surgical field of view, further compounds the difficulties of using an FGS agent effectively. Surgical resection of primary NETs is most commonly performed in the pancreas, small intestine, or lungs. Therefore, low accumulation of
Ga-MMC(IR800)-TOC in these tissues is necessary in order to generate suitable contrast for determining surgical margins or lymph node metastases. Macroscopic ex vivo imaging showed that our agent can provide contrasts of >2 in pancreas and lungs, and >4.5 in muscle and small intestine. Importantly, these TBRs were acquired without dose optimization or an accompanying imaging device. Suitable contrast ratios for FGS are difficult to define given the increasing number of drug-device combinations in the OR. However, our results compare favorably to pre-clinical and clinical TBRs during fluorescence-guided resection of tumors (34, 39, 115) and indicate potential for intraoperative visualization of NET deposits using Ga-MMC(IR800)-TOC.

Previous work by our group showed SSTR2-mediated uptake of a $^{64}$Cu labeled MMC(IR800)-TOC analog using in vitro and in vivo studies, where competition with octreotide strongly reduced uptake in SSTR2 expressing tumors (57). In addition, a probe variant with a scrambled peptide showed very low tumor uptake. The present study further confirms the intact in vivo receptor specificity provided by TOC as shown by clear differences in tumor fluorescence between HCT116-SSTR2 and HCT116-WT tumors macroscopically (in vivo, ex vivo) and microscopically. Additionally, a non-targeted analog showed decreased tumor uptake.

Identifying the optimal injection dose can also attain improved image contrast. In the case of radiotracers, both labeled (i.e., $^{68}$Ga-DOTA-TOC) and unlabeled (i.e., DOTA-TOC) species are present upon administration and could result in competition for binding sites and reduce radioactive signal in tumors. Fluorescent agents generally consist of a single chemical species and do not experience such effects. Therefore, optical probes may not necessarily need to adhere to microdoses and may benefit from increased agent dosage. Rosenthal et al. previously showed that following the administration of 2.5, 25 and 62.5 mg cetuximab-IR800/m$^2$, the TBRs of resected human tumors using closed-field imaging were highest with the middle dose (34).

Since larger doses may also produce more non-targeted uptake that reduces contrast and negatively affects specificity, we examined the correlation between injected dose and absolute fluorescence by injecting xenografts with 0.5 or 2 nmol of Ga-MMC(IR800)-TOC. Although the 2 nmol dose produced a nearly 4-fold increase in tumor fluorescence, TBRs were
all higher with the low-dose cohort. This is likely attributable to non-specific binding of Ga-MMC(IR800)-TOC that is amplified at higher doses and suggests more effective tumor visualization at lower injection amounts. Further dose optimization studies could identify maximal TBRs that reduce the potential of false-positives and enhance predictive value and diagnostic accuracy.

Recently, there has been a surge of interest in evaluating tumor margins with surgical specimen mapping. This strategy consists in utilizing closed-field devices with a controlled environment for back-table fluorescence imaging of surgical resections. This method has been proposed as a complementary tool during in situ surgical navigation for screening intraoperative margin samples and prioritizing the fluorescent ones for further examination. Clinical studies have effectively integrated ex vivo macroscopic imaging as a validation method with implications for fluorescence-guided pathology. In one study, the accumulation of panitumumab-IR800 in surgical samples highly correlated with tumor location with sensitivities and specificities >89%, while the NIRF signal predicted the distance of tumor tissue to the cut surface of the specimen (115). In another study with bevacizumab-IR800, an analytical framework for correlating intraoperative fluorescence signals with histopathology was applied and demonstrated an 88% increase in detection rates of tumor-involved margins (116). In order to examine tissue mapping with Ga-MMC(IR800)-TOC, we correlated SSTR2 expression via IHC with H&E staining and NIRF signal accumulation in frozen sections prepared from mouse xenografts. While H&E provides boundary demarcation of tumor from non-tumor tissue, IHC identifies and determines the distribution of SSTR2 expression. Thus, this histopathological combination serves as a method for assessing the binding and delineation specificity of Ga-MMC(IR800)-TOC. Frozen section analysis of HCT116-SSTR2 xenografts treated with Ga-MMC(IR800)-TOC revealed fluorescent delineation of tumor from non-tumor tissue in excellent agreement with tumor morphology and expression status determined by H&E and IHC staining, respectively. Conversely, negligible signal was produced from the SSTR2-deficient tumor and healthy tissues. These results showed the potential clinical effectiveness of Ga-MMC(IR800)-TOC for specifically
delineating tissues based on the presence of SSTR2 and indicate promising potential for image-guided NET pathology.

In summary, we demonstrated for the first time that an SSTR2-targeted FGS agent can be used for highly specific tumor targeting. Importantly, we showed an excellent strategy for the intact retention of agonist-induced mechanisms and in vitro/in vivo receptor-targeting capabilities upon dye conjugation to the most commonly used SSA for PET imaging in NETs. Our results also showed the effectiveness of the MMC technology for combining fluorescent and nuclear contrast into a single targeted probe. We presented the advantages of applying the radioactive utility of the MMC for cross-validating and quantifying the pharmacokinetics of the resulting FGS agent. Finally, imaging at the macro, meso and microscopic scales provided comprehensive validation of receptor-mediated uptake and strongly indicate translational utility for FGS in NETs.

**Future Directions**

There are two major areas that we are actively looking to investigate further. The first one involves the determination of a strategy for significantly reducing the prominent liver signal that we have observed up to 24 h. A decrease in liver signal could potentially allow the delineation of SSTR2 overexpressing lesions in the hepatic system. This interest arises from the well-established role of neuroendocrine liver metastases that largely contribute to mortality rates in NET patients. To put it into context, more than 40% of NET patients develop metastatic liver burden over the course of the disease and have a 5-year survival rate ranging from 13 to 54% (69). Our initial assessment will focus on further optimizing the injected dose and extending the time for imaging relative to agent administration. The results of this study will provide insight to the question “how low can we go and still acquire relevant TBRs?” at sites of NET formation. Additionally, we will determine whether Ga-MMC(IR800)-TOC either requires more time for clearance from the liver (i.e., signal at 24 h vs. 48 h) or whether dye conjugation causes irreversible liver retention. Subsequently, we are interested in substituting the highly anionic IR800 for a NIRF dye with comparable optical properties, but with reduced anionic burden and a
well-balanced charge distribution over its surface (i.e., ZW800-1). We hypothesize that this switch could improve the pharmacokinetic disposition of our FGS agent by means of faster elimination from non-target tissues, while also reducing non-specific signal accumulation.

The second area that we are looking to further investigate is the sensitivity and specificity of our FGS agent using a metastatic animal model. Clinically, SSTR2 overexpressing malignancies will continue to be diagnosed using a gold standard. Importantly, patients that have been identified as candidates for surgery using $^{68}$Ga-DOTA-TOC or -TATE, would be candidates for FGS with Ga-MMC(IR800)-TOC. Thus, it is critical to evaluate the ability of our FGS agent to target tumor deposits that have been previously identified using PET/CT with $^{68}$Ga-DOTA-TOC. The results of this study will complement our strong in vivo specificity data and provide the groundwork prior to transitioning to imaging larger animals (i.e., pigs) with $^{68}$Ga-DOTA-TOC and Ga-MMC(IR800)-TOC.

Altogether, these findings will also serve as the basis and benchmark as we transition towards the application of Ga-MMC(IR800)-TOC in other disease models with SSTR2 overexpression. Several tumors of the nervous system have been shown to express SSTR2 at high density (77). Specifically, studies have shown that SSTRs are overexpressed in more than 90% of neuroblastoma (117) and medulloblastoma (118) tumors in children, while clinical studies continue to investigate their implication for diagnosis and therapy (119). Recently, Dijkstra et al. reported the establishment of SSTR2 as the “most promising receptor for meningioma targeting” (120) with excellent potential for intraoperative imaging using targeted contrast agents. Thus, candidates for surgery within this patient population could benefit from FGS with Ga-MMC(IR800)-TOC.

Finally, given versatility of the MMC technology, we are interested in expanding the role of our radioactive linker for the assessment of not only targeting approaches beyond TOC (i.e., other peptides, small molecules), but for also evaluating other therapeutic strategies. The conjugation of a reporter or payload to the MMC occurs through copper-free click chemistry – an ever-growing field that facilitates the development of targeted probes through incorporation of
reactive groups for facile bioconjugation. Therefore, the practicality of a “plug-and-play” platform via the MMC provides an efficient method for the rapid synthesis and investigation of systemic therapies including peptide-drug conjugates (i.e., with MMAE) and photodynamic therapy (i.e., with IR700DX). These theranostic agents could be a valuable alternative for patients with advanced stages of SSTR2-positive metastases, where surgery may not be an option.
Bibliography


Vita

Servando Hernandez Vargas was born in Maracaibo, Zulia, Venezuela, the son of Dr. Eglee Josefina Vargas Acosta and Giovanni Rene Hernandez Gonzalez. After completing his work at Colegio Aleman de Maracaibo, Maracaibo, Zulia, Venezuela in 2007, he entered the University of Houston in Houston, Texas. He received the degree of Bachelor of Science with a major in biomedical engineering from the University of Houston in August, 2013. For the next two years, he worked as an engineering consultant in the oil & gas industry. In August of 2016 he entered The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences.

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