Synthesis of a Fluorescently Labeled ^{68}Ga -DOTA-TOC Analog for Somatostatin Receptor Targeting

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S Supporting Information

ABSTRACT: Fluorescently labeled imaging agents can identify surgical margins in real-time to help achieve complete resections and minimize the likelihood of local recurrence. However, photon attenuation limits fluorescence-based imaging to superficial lesions or lesions that are a few millimeters beneath the tissue surface. Contrast agents that are dual-labeled with a radionuclide and fluorescent dye can overcome this limitation and combine quantitative, whole-body nuclear imaging with intraoperative fluorescence imaging. Using a multimodality chelation (MMC) scaffold, IRDye 800CW was conjugated to the clinically used somatostatin analog, ^{68}Ga -DOTA-TOC, to produce the dual-labeled analog, ^{68}Ga -MMC(IRDye 800CW)-TOC, with high yield and specific activity. *In vitro* pharmacological assays demonstrated retention of receptor-targeting properties for the dual-labeled compound with robust internalization that was somatostatin receptor (SSTR) 2-mediated. Biodistribution studies in mice identified the kidneys as the primary excretion route for ^{68}Ga -MMC(IRDye 800CW)-TOC, along with clearance via the reticuloendothelial system. Higher uptake was observed in most tissues compared to ^{68}Ga -DOTA-TOC but decreased as a function of time. The combination of excellent specificity for SSTR2-expressing cells and suitable biodistribution indicate potential application of ^{68}Ga -MMC(IRDye 800CW)-TOC for intraoperative detection of SSTR2-expressing tumors.

KEYWORDS: Dual labeling, PET, NIRF, somatostatin receptor

Intraoperative imaging is an emerging field that uses targeted fluorescent contrast agents to distinguish tumor from nontumor tissues.^{1,2} These agents consist of a targeting moiety and a fluorescent dye, and have been shown to significantly improve tumor detection in patients when compared to visual inspection alone.³ Importantly, fluorescence-guided surgery (FGS) can identify surgical margins in real-time and could potentially increase the prospects of performing complete resections and minimize the likelihood of local recurrence.^{4,5} This may, in turn, reduce the need for additional surgical procedures and limit excessive removal of healthy tissues and tissue components that are vital to physiologic function (i.e., blood vessels, nerves, lymph nodes). Accordingly, several clinical trials are investigating the intraoperative utility of fluorescently labeled antibodies, peptides, and small molecules, highlighting the importance of FGS in cancer treatment.⁶

Despite these characteristics, photon attenuation limits fluorescence-based imaging to superficial lesions or lesions

that are few millimeters beneath the tissue surface. Contrast agents that are dual-labeled with a radionuclide and fluorescent dye can overcome this limitation and combine quantitative, whole-body nuclear imaging with intraoperative fluorescence imaging.^{7–9} Unlike the coadministration of separately labeled fluorescent and radioactive compounds, which may possess significantly different physiochemical and pharmacokinetic properties, dual labeling ensures that signals from each reporter originate from the same location at the same time. Other key advantages of dual labeling include: eliminating the need for a surrogate to quantify tissue uptake, characterizing one chemical entity, and possessing an intrinsic method for cross-validation.¹⁰ Clinical translation of nontargeted, dual-labeled nanocolloids containing indocyanine green and $^{99\text{m}}\text{Tc}$ have shown that it is

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Scheme 1. Synthesis of Monosubstituted MMC Intermediate (4)

Scheme 2. Synthesis of Azido-MMC Intermediate (7) for Solid-Phase Peptide Synthesis

possible to perform surgical planning and surgical navigation with a single agent,¹¹ and further highlights the potential impact of targeted dual-labeled agents.

An ideal approach for developing a dual-labeled agent would be to use a clinically established radiotracer since it could provide a benchmark for characterization of the dual-labeled counterpart.¹² Synthetic peptides that target somatostatin receptor (SSTR) overexpression have been widely used to image neuroendocrine tumors (NETs)¹³ and are the most well-characterized molecular imaging model system. Tyr3-octreotide, or TOC, is an octapeptide that has been extensively used for developing radiolabeled peptide conjugates for positron emission tomography (PET),^{14,15} while also undergoing dye labeling for optical imaging.¹⁶ Dual-labeled TOC analogs, however, have proven significantly more challenging to develop and have yet to result in a compound that retains key characteristics of the parent radiotracers such as high receptor specificity and favorable pharmacokinetics. Moreover, there are no reports, to the best of our knowledge, on the development of a bioactive fluorescent analog of the most commonly used PET tracer for NET imaging, ⁶⁸Ga-DOTA-TOC (or ⁶⁸Ga-DOTA-TATE, analog with C-terminal acid). Here, we used a

modular dual labeling scaffold, referred to as a multimodality chelator (MMC),¹⁷ to synthesize a dual-labeled TOC analog, and evaluated agent properties *in vitro* and *in vivo*.

We aimed to develop a fluorescent ⁶⁸Ga-DOTA-TOC analog with minimal structural deviations from the parent compound in order to maximize the retention of binding characteristics. It should be noted that multiple dual labeling scaffolds have previously been reported in the literature, but none are capable of maintaining the chelator–peptide orientation of DOTA-TOC. In comparison to the MMC, they possess significant technical limitations, which include a peptide-based scaffold that can employ a limited range of chemistries,¹⁸ and chelator-based approaches with diethylenetriaminepentaacetic acid¹⁹ and a sarcophagine analog²⁰ that are structurally different than DOTA and are not ideal for ⁶⁸Ga chelation due to their coordination properties. Accordingly, we selected the macrocyclic compound 1,4,7,10-tetraazacyclododecane-1,7-bis(*t*-butyl acetate) (DO2A) to enable selective functionalization that mimics the chelator–peptide footprint of DOTA-TOC. Starting with L-glutamic acid γ -benzyl ester (1), the azido pendant arm 3 was synthesized according to Scheme 1 (detailed conditions are provided in the Supporting Information) and conjugated to

DO2A to produce the monosubstituted MMC-intermediate 4. Careful purification of the reaction mixture by column chromatography allowed for separation of the mono- and disubstituted products. To produce the azido-MMC intermediate for solid-phase conjugation to TOC, 7 was prepared as shown in Scheme 2. Next, standard Fmoc/*t*Bu chemistry was used to synthesize TOC, and conjugation of 7 to the N-terminus of the resin-bound peptide was carried out via *in situ* aminium-based activation (Scheme 3). Protecting groups were

Scheme 3. Synthesis of ^{68}Ga -MMC(IRDye 800CW)-TOC (9)

removed from the MMC and the side chains of the peptide in a single step with TFA, and the thiol groups were oxidized to form 8. The reaction of 8 with IRDye 800-DBCO yielded the fluorescent compound MMC(IRDye 800CW)-TOC.

We selected ^{68}Ga to permit direct comparison of the dual-labeled analog to ^{68}Ga -DOTA-TOC. The 68 min half-life of ^{68}Ga is compatible with the pharmacokinetic profile of peptide-

based agents and, in combination with its availability in generator form and established radiolabeling protocols, makes it an attractive radionuclide for dual-labeled peptide development. Moreover, the use of ^{68}Ga permits quantitative PET imaging and is aligned with current clinical practices for neuroendocrine tumors. For the radiolabeling experiments, ^{68}Ga was eluted from a generator, concentrated on a cation exchange cartridge, and eluted with an acidified acetone solution.²¹ The radioactive solution was added to 20 nmol of MMC(IRDye 800CW)-TOC, DOTA-TOC, or nontargeted MMC(IRDye 800CW) in 0.2 M NaOAc, and the reactions were heated at 95 °C for 15 min. ^{68}Ga -MMC(IRDye 800CW)-TOC was obtained in $79.9 \pm 8.1\%$ radiochemical yield (nondecay-corrected) and with >99% radiochemical purity following purification with a C-18 cartridge. High specificity activity (87.3 TBq/mmol, 2360 Ci/mmol) suggested minimal impact of dye conjugation on the chelation properties of the macrocycle. Nonradioactive analogs for pharmacological and fluorescence studies were synthesized according to methods used for the radiolabeled agents.

A major challenge with dual-labeled agents is retaining the binding properties of the peptide-conjugate after attachment of the dye. This was observed in a recent study where conjugation of Cy5 to ^{111}In -DTPA-octreotide caused a significant reduction in receptor affinity and internalization rate.²² The use of near-infrared (NIR) dyes, which offer increased depth penetration but are themselves comparable in size to the peptide-conjugate, further amplifies this effect and may limit specificity for receptor-targeting. In our approach, the MMC was designed to maximize the distance between the dye and peptide to reduce steric interference and retain the binding characteristics of ^{68}Ga -DOTA-TOC. To identify the effects of dye conjugation on receptor pharmacology, SSTR2-expressing HEK-293 cells were used to measure potency for inhibiting cyclic adenosine monophosphate (cAMP) formation and stimulating receptor internalization (experimental details in the Supporting Information). Using Ga-DOTA-TOC as a reference standard, we found that Ga-MMC(IRDye 800CW)-TOC was able to inhibit NHK477 (water-soluble forskolin derivative)-stimulated cAMP formation with high efficacy (Figure 1a). Both agents demonstrated maximum possible inhibition of cAMP, and an EC₅₀ value of 0.066 ± 0.012 nM was obtained for Ga-MMC(IRDye 800CW)-TOC, which despite the significant bulk added to the agent by IRDye 800CW, was comparable to Ga-DOTA-TOC (0.009 ± 0.001 nM) and still in the low nanomolar range. Since binding of TOC to SSTR2 causes

Figure 1. Potency of peptide conjugates for (a) cAMP inhibition and (b) receptor internalization in HEK293-SSTR2 expressing cells.

internalization of the receptor–ligand complex, we also examined the potency of Ga-MMC(IRDye 800CW)-TOC for inducing receptor internalization to further verify retention of agonist properties after dye conjugation. SSTR2-expressing HEK-293 cells were incubated with increasing amounts of Ga-MMC(IRDye 800CW)-TOC and Ga-DOTA-TOC, and cell surface receptor levels were measured by enzyme-linked immunosorbent assay (ELISA) using published procedures.²³ As shown in Figure 1b, Ga-MMC(IRDye 800CW)-TOC effectively stimulated 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 provided evidence that the MMC scaffold could be used to prepare a fluorescent DOTA-TOC analog with intact pharmacological properties.

Next, we used confocal microscopy to examine the receptor-targeting properties of Ga-MMC(IRDye 800CW)-TOC in HCT116 cells that stably overexpress SSTR2 (HCT116-SSTR2). After a 1 h incubation, Ga-MMC(IRDye 800CW)-TOC was strongly internalized in HCT116-SSTR2 cells, but not in HCT116-WT cells, which lack the receptor (Figure 2a).

Figure 2. Confocal microscopy showing (a) uptake and internalization and (b) blocking of MMC(IRDye 800CW)TOC in HCT116-SSTR2 and wild type HCT116 cells. Red, Ga-MMC(IRDye 800CW)-TOC; blue, Hoechst 33342 nuclear stain.

For validation, receptor expression was confirmed via an anti-SSTR2 antibody, which showed staining in HCT116-SSTR2 cells, while no receptor expression was seen in wild-type cells (Supporting Figure 3). To further demonstrate specificity, 10- and 100-fold excesses of octreotide were used to block peptide binding sites prior to incubation with Ga-MMC(IRDye 800CW)-TOC. For both blocking doses, a nearly complete elimination of Ga-MMC(IRDye 800CW)-TOC uptake was observed, providing additional evidence for high specificity of SSTR2-mediated uptake of the dual-labeled agent (Figure 2b).

Cellular uptake and blocking of the radiolabeled analogs were then investigated according to published protocols.²⁴ As shown in Figure 3a, $25.0 \pm 1.7\%$ of ^{68}Ga -MMC(IRDye 800CW)-TOC

Figure 3. Cellular uptake (a) and blocking (b) of peptide conjugates in HCT116-SSTR2 and wild type HCT116 cells. * $P < 0.0001$.

was taken up by HCT116-SSTR2 cells at 1 h. This value was similar to ^{68}Ga -DOTA-TOC ($21.5 \pm 3.7\%$) and further suggests that dye conjugation did not impact SSTR2-targeting capabilities. To assess any nonspecific binding that may be attributable to the MMC-dye complex, cells were also incubated with nontargeted ^{68}Ga -MMC(IRDye 800CW) and showed no notable association with tumor cells. Parallel studies with HCT116-WT cells showed comparatively low uptake for ^{68}Ga -MMC(IRDye 800CW)-TOC and ^{68}Ga -DOTA-TOC, further indicating SSTR2 specificity. Blocking with increasing amounts of octreotide produced a dose-dependent reduction in uptake that correlated with the fluorescent microscopy findings (Figure 3b). Using a 10-fold excess of octreotide, ^{68}Ga -MMC(IRDye 800CW)-TOC uptake was reduced by $78.5 \pm 9.6\%$ ($P < 0.0001$) and by $93.7 \pm 1.6\%$ ($P < 0.0001$) with a 100-fold excess of the competitive inhibitor. From the *in vitro* experiments, we showed highly complementary data, which demonstrate the utility of the MMC scaffold for producing a bioactive dual-labeled agent.

Since the excellent clinical imaging characteristics of ^{68}Ga -DOTA-TOC are attributable to the combination of high SSTR2 specificity and low background signal, we then examined the effects of dye labeling on pharmacokinetics in healthy mice. Uptake values were obtained at 15 min, 1 h, and 3 h postinjection and identified the kidneys as the primary excretion route for both agents (Figure 4). This observation suggests that, despite the increase in size and the presence of

Figure 4. Tissue uptake values for ^{68}Ga -MMC(IRDye 800CW)-TOC and ^{68}Ga -DOTA-TOC in healthy mice.

multiple aromatic groups on IRDye 800CW, ^{68}Ga -MMC (IRDye 800CW)-TOC retains the hydrophilic properties of a conventional radiolabeled peptide and is cleared similarly. Dye labeling did cause higher clearance via reticuloendothelial system (RES) organs (liver and spleen) and longer retention in blood, indicating some increase in lipophilicity. These values, however, decreased following the blood-pool phase and may potentially provide adequate tumor contrast in the abdomen. This is consistent with a recent study on Cy5-labeled ^{111}In -DTPA-octreotide which showed that signal from the dual-labeled analog was higher in nontumor tissues compared to ^{111}In -DTPA-octreotide, suggesting a moderate increase in non-specific binding after dye labeling.²² Other notable differences were observed in the pancreas, where ^{68}Ga -DOTA-TOC uptake was approximately 2-fold higher than ^{68}Ga -MMC (IRDye 800CW)-TOC ($P < 0.05$), and in the lung, where ^{68}Ga -MMC (IRDye 800CW)-TOC signal was persistent up to 3 h. In the remaining tissues, radioactivity decreased over time for both agents and demonstrate that a suitable pharmacokinetic profile is achievable following dye conjugation.

In conclusion, we used the MMC scaffold to develop a fluorescently labeled ^{68}Ga -DOTA-TOC analog with excellent specificity for SSTR2-expressing cells. Although dye conjugation more than doubled the weight of the parent compound, our drug design strategy enabled retention of receptor binding properties as shown by independent pharmacologic, fluorescent, and radioactive-based *in vitro* assays. We also showed that ^{68}Ga -MMC (IRDye 800CW)-TOC is predominantly cleared through the kidneys and possesses a reasonable *in vivo* pharmacokinetic profile that could be further improved through MMC optimization. Importantly, we presented dual labeling methods that used conventional solid-phase and radiolabeling protocols, making the MMC suitable for use with peptides that target tumor-associated receptors beyond SSTRs. Further *in vivo* characterization in translationally relevant tumor models is needed to evaluate the effectiveness of ^{68}Ga -MMC (IRDye 800CW)-TOC for fluorescence-guided surgery.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.7b00125.

Experimental details for the synthesis all MMC compounds with supporting ESI-MS, NMR, and HPLC data. Methods for radiolabeling and cold Ga labeling. Full experimental procedures for *in vitro* studies, and results for fluorescence imaging. Methods for biodistribution study (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

SSTR, somatostatin receptor; FGS, fluorescence-guided surgery; NETs, neuroendocrine tumors; TOC, Tyr3-octreotide; PET, positron emission tomography; MMC, multimodality chelator; DO2A, 1,4,7,10-tetraazacyclododecane-1,7-bis(t-butyl acetate); NIR, near-infrared; cAMP, inhibiting cyclic adenosine monophosphate; ELISA, enzyme-linked immunosorbent assay; TBTA, *tert*-butyl 2,2,2-trichloroacetimidate; DMA, *N,N*-dimethylacetamide; EDCI, *N*-(3-(dimethylamino)propyl)-*N'*-ethylcarbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole hydrate; DIEA, *N,N*-diisopropylethylamine

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