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Delta-Opioid Receptor (δ OR) Targeted Near-Infrared Fluorescent Agent for Imaging of Lung Cancer: Synthesis and Evaluation *In Vitro* and *In Vivo*

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Abstract

In the United States, lung cancer is the leading cause of cancer deaths and ranks second in the number of new cases annually among all types of cancers. Better methods or tools for diagnosing and treating this disease are needed to improve patient outcomes. The delta opioid receptor (δ OR) is reported to be overexpressed in lung cancers and not expressed in normal lung. Thus, we decided to develop a lung cancer-specific imaging agent targeting this receptor. We have previously developed a δ OR-targeted fluorescent imaging agent based on a synthetic peptide antagonist (Dmt-Tic) conjugated to a Cy5 fluorescent dye. In this work, we describe the synthesis of Dmt-Tic conjugated to a longer wavelength near-infrared fluorescent (NIRF) dye, Li-cor IR800CW. Binding affinity of Dmt-Tic-IR800 for the δ OR was studied using lanthanide time-resolved fluorescence (LTRF) competitive binding assays in cells engineered to overexpress the δ OR. In addition, we identified lung cancer cell lines with high- and low-endogenous expression

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Supporting Information Description

Analytical RP-HPLC chromatogram for Dmt-Tic-IR800, ESI-MS spectrum for Dmt-Tic-IR800, optical characterization of Dmt-Tic-Lys-Cy5 and Dmt-Tic-IR800, mRNA microarray data for the expression of δ OR in lung cancer cell lines, dose determination for Dmt-Tic-IR800 using engineered and endogenous cells *in vivo*, and imaging of Dmt-Tic-IR800 in engineered cells and endogenous lung cancer cells *in vivo* are provided as supplemental material. This material is available free of charge via the Internet at <http://pubs.acs.org>.

of the δ OR. We confirmed protein expression in these cell lines using confocal fluorescence microscopy imaging and used this technique to estimate the cell-surface receptor number in the endogenously expressing lung cancer cell lines. The selectivity of Dmt-Tic-IR800 for imaging of the δ OR *in vivo* was shown using both engineered cell lines and endogenously expressing lung cancer cells in subcutaneous xenograft models in mice. In conclusion, the δ OR-specific fluorescent probe developed in this study displays excellent potential for imaging of lung cancer.

Graphical abstract

Introduction

Lung cancer is the leading cause of cancer deaths and ranks second in the number of new cancer cases annually among all types of cancer in men and women in the United States.¹ The five-year survival rate for this cancer is low.² Thus, there is a need for improved methods for diagnosing and treating this disease. The goal of this work was to develop a targeted fluorescent agent for the imaging of lung cancer. Fluorescently labeled targeted agents can potentially be used for real-time surgical guidance through the use of endoscopic instruments with fluorescence capability. In cases where lung-conservation is imperative, real-time fluorescence imaging using tumor-specific molecular imaging agents could enable the detection and removal of residual disease during surgery and profoundly affect the course of treatment by reducing the number of patients with incomplete resections. Targeted fluorescent agents could also be used to identify mediastinal lymph nodes for staging during surgery or for early detection of malignant lesions by fluorescence bronchoscopy.

Molecular imaging is a rapidly growing field whose utility has been demonstrated in numerous applications and thus its importance has been increasingly recognized. In oncology, molecular imaging is used routinely in both research and clinical settings.^{3–6} In the research setting, a variety of molecular imaging modalities are used with the chosen technique depending on the specific application, since each one has its own advantages and limitations. In the clinic, molecular imaging with ¹⁸F-fluorodeoxyglucose (FDG) for positron emission tomography (PET) has been widely used. More recently, a number of other modalities, such as optical imaging (fluorescence), magnetic resonance imaging (MRI), computed tomography (CT) and ultrasound (US), have been applied to clinical

molecular imaging and new reagents for these applications are continuously being developed.³ Fluorescence imaging has been found effective for several clinical applications including intraoperative guidance.^{7–16} Fluorescence has the advantages of high sensitivity, ease of use, cost-effectiveness, and a lack of ionizing radiation.^{3, 4} Hence, a goal of fluorescence imaging in oncology is to differentiate the cancer tissue from the surrounding normal tissue. The two main methods for achieving this selectivity are through activatable or targeted fluorescent agents.^{5, 6, 14} Thus, an important aspect in the development of novel imaging agents is the selection of markers, such as enzymes for activatable agents^{17–23} or receptors for targeted agents^{7, 15, 16, 24–32}, which could differentiate neoplastic tissue from normal tissue.

The delta opioid receptor (δ OR) is a member of the G-protein coupled receptor family that is involved in various normal physiological processes.^{33, 34} It is also reported to play a role in several diseases including cancer.^{35, 37} The δ OR is overexpressed in lung cancer and not expressed in normal lung.³⁸ Its expression has been demonstrated in human lung cancer cell lines using ligand binding

assays

.^{39, 40} In addition, there are several previous studies describing the use of PET and single-photon emission computed tomography (SPECT) agents.

Our group has previously synthesized a δ OR-targeted fluorescent imaging agent with low nM affinity based on a synthetic peptide antagonist (Dmt-Tic)⁴³ conjugated to a Cy5 fluorescent dye (Dmt-Tic-Lys-Cy5).⁴⁴ This agent was evaluated using a colorectal cancer cell line (HCT-116) engineered to express the δ OR.⁴⁵ It was shown to have high δ OR binding affinity *in vitro*, demonstrated selectivity for the δ OR *in vitro* and *in vivo*, and exhibited good pharmacokinetic and biodistribution profiles *in vivo*.⁴⁴ Based on these studies, we hypothesize that Dmt-Tic would have demonstrable potential as a δ OR-targeting ligand for the imaging of lung cancer. To improve its potential for *in vivo* imaging and clinical translation, in the current study we conjugated Dmt-Tic to a near-infrared fluorescent (NIRF) dye (Li-cor IR800CW) with longer excitation and emission wavelengths than the previously reported Cy5 dye. *In vivo* fluorescence imaging with NIR dyes have decreased background signal from autofluorescence and less absorption and scattering of the excitation and emission light compared to shorter wavelength dyes. In addition, this agent would be suited for use with the currently commercially available clinically used real-time fluorescence instruments that detect at longer (NIRF) wavelengths. In this paper, we describe the synthesis of the novel Dmt-Tic-IR800 agent and the evaluation of this agent for imaging of the δ OR both *in vitro* and *in vivo*.

Results and Discussion

Synthesis and Characterization of Dmt-Tic-IR800

Our group has previously described the synthesis of a δ OR-targeted fluorescent agent (Dmt-Tic-Lys-Cy5) based on a small synthetic peptide antagonist Dmt-Tic⁴³ (Dmt: 2',6'-dimethyl-L-tyrosine; Tic: 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) conjugated to a fluorescent dye (Cy5) via a small linker to the side chain of lysine (Lys).^{44, 46} This probe was shown to have high binding affinity for the δ OR ($K_i = 3$ nM), high inhibitory potency ($K_e = 37$ pM), and good *in vivo* selectivity, pharmacokinetics and biodistribution profiles.⁴⁴

In order to improve the characteristics of this probe for future clinical applications, in this work, we synthesized a novel imaging agent using the same targeting moiety (Dmt-Tic) but conjugated it to a longer wavelength near-infrared fluorescent (NIRF) dye, Li-cor IR800CW. We have chosen the Li-cor IR800CW dye for these studies because it has excitation and emission wavelengths above 750 nm, resulting in less background due to decreased tissue absorbance and autofluorescence at this wavelength. This will result in improved tissue penetration. In addition, Li-cor IR800CW is a derivative of the FDA approved indocyanine green (ICG) fluorescent dye, has been shown to be non-toxic,⁴⁷ and is available with GMP quality enabling an easier transition to the clinic.

We synthesized Dmt-Tic-IR800 using a synthetic strategy similar to the one described previously (Scheme 1).^{44, 46} We chose to use the same attachment point and linker as in our previously described Dmt-Tic-Lys-Cy5 agent as they were shown not to interfere with binding affinity or selectivity for the δ OR. The peptide, Dmt-Tic-Lys-OH, was synthesized on solid phase according to our published procedure. A 3-mercaptopropionyl (Mpr) linker was conjugated to the side chain of the lysine residue of the peptide in order to enable conjugation of the IR800CW dye via thiol-maleimide chemistry. Following cleavage of the peptide-linker conjugate from the resin and purification, the dye conjugation was carried out in solution to afford the final product (Compound 1, Dmt-Tic-IR800, Scheme 1).

To demonstrate the improved *in vivo* imaging potential of the novel Dmt-Tic-IR800 compound in relation to the previously reported Dmt-Tic-Lys-Cy5 agent, we characterized and compared the optical properties of the two probes. We measured the absorbance (Figures S3A and S3B) and emission (Figure S3C and S3D) spectra of the fluorescent imaging agents. The absorbance and emission maxima of Dmt-Tic-Lys-Cy5 are 650 nm (Figure S3A) and 676 nm (Figure S3C) which are similar to the reported absorbance and emission maxima of the free Cy5 dye at 649 nm and 670 nm, respectively. Thus, conjugation of the Cy5 dye to the Dmt-Tic targeting ligand does not affect these properties. For Dmt-Tic-IR800, the absorbance maximum, 778 nm (Figure S3B), is similar to that reported for the free IR800CW dye, 774 nm. However, the emission maximum of IR800CW is shifted upon conjugation to the Dmt-Tic targeting ligand with the emission maximum for Dmt-Tic-IR800 being 832 nm (Figure S3D) while that for the free IR800CW dye is 789 nm. Hence, the Stokes-shift increased from 15 nm for free IR800CW to 54 nm for the conjugate. A longer Stokes-shift is desirable for imaging as the range of wavelengths used for excitation can be greater and the range of emission signal detected can be greater without overlap. As the maximum absorbance and emission wavelengths for Dmt-Tic-IR800 are above 750 nm and longer than those for Dmt-Tic-Lys-Cy5, the Dmt-Tic-IR800 agent is better suited for *in vivo* imaging applications as there is less absorption and autofluorescence from biological molecules, enhanced tissue penetration of light and less photon scatter at these wavelengths. We also studied the photobleaching of Dmt-Tic-Lys-Cy5 and Dmt-Tic-IR800 (Figure S3E). The Dmt-Tic-Lys-Cy5 agent photobleaches as indicated by the significant differences in the percent of the initial fluorescence intensity when the agent is incubated in the light versus when it is kept in the dark ($p = 0.0001$ at time = 3 hours). We observed a decrease in the percent of the initial fluorescence intensity for the samples even when incubated in the dark due to repeated excitation of the fluorophores over the course of the experiment. In contrast,

the percent of the initial fluorescence intensity remaining for Dmt-Tic-IR800 incubated in the dark is not significantly different than the percent of the initial fluorescence intensity remaining for Dmt-Tic-IR800 incubated in the light except at longer incubation times (greater than or equal to 7 hours). Even though it is significant at these longer incubation times, the difference in percent of the initial fluorescence intensity for agent incubated in the light versus in the dark is small (~6–13%). Thus, we conclude that Dmt-Tic-IR800 is better suited for *in vivo* imaging and clinical translation.

As mentioned above, we have previously shown that Dmt-Tic binds with high affinity to the δ OR and this binding affinity is retained upon conjugation to a fluorescent dye (Cy5).⁴⁴ To test the binding of Dmt-Tic-IR800 to the δ OR, we performed lanthanide time-resolved fluorescence (LTRF) competition binding assays.^{48, 49} This assay uses whole cells, rather than membrane preparations, and thus the only receptors available for binding are those present on the cell surface. Our lab has previously generated an engineered cell line that stably overexpresses the δ OR based on the colorectal cancer cell line HCT-116.⁴⁵ We have demonstrated that the parental HCT-116 cell line lacks endogenous expression of the δ OR and thus does not bind to the δ OR-targeted ligands. In addition, using *in cyto* time-resolved fluorescence saturation binding assays, we have determined the receptor number for the HCT-116/ δ OR engineered cell line to be $1.61 \times 10^6 \pm 1.10 \times 10^5$ δ OR/cell.^{48, 49} For the LTRF competition binding assays with Dmt-Tic-IR800, we used the engineered HCT-116/ δ OR cell line since it has a high number of receptors on the cell surface. Cells were incubated with europium-labeled delta opioid receptor agonist (Eu-DTPA-DPLCE) and increasing concentrations of Dmt-Tic-IR800 as the competing ligand. Higher concentrations of Dmt-Tic-IR800 resulted in a decreased signal from the europium-labeled ligand due to competition for binding to the δ OR (Figure 1). We obtained an average K_i of 1.43 ± 0.24 nM which is similar to that of the Dmt-Tic-Lys-Cy5 ligand, thus verifying that Dmt-Tic-IR800 retains high δ OR binding affinity.

Characterization of δ OR Expression in Cell Lines

In order to study our δ OR-targeted imaging agent, we first had to identify δ OR-expressing cell lines that could be used for *in vitro* and *in vivo* experiments. As mentioned above, our lab has previously generated an engineered cell line that stably overexpresses the δ OR, HCT-116/ δ OR.⁴⁵ This engineered cell line is useful as a model system since the cells are adherent and are capable of forming xenografts in mice. However, the goal of this work is to design lung cancer targeted imaging agents and hence, we sought to identify a lung cancer cell line that contains these characteristics and has endogenous expression of the δ OR. We analyzed mRNA expression microarray data for *OPRD1*, the gene that encodes the δ OR, in a panel of lung cancer cell lines (Figure S4). From this screen, we identified DMS-53, a small cell carcinoma cell line, as having high mRNA expression and H1299, a large cell neuroendocrine cell line, as having little or no *OPRD1* mRNA expression (Figure 2A). To further confirm these results, we screened these two lung cancer cell lines using quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) for expression of *OPRD1*. We also performed qRT-PCR on the parental HCT-116 and HCT-116 engineered to overexpress δ OR as negative and positive controls, respectively. In agreement with the microarray data, we observed that DMS-53 was positive for expression of *OPRD1* and

H1299 was negative (Figure 2B). In addition, we compared the expression of *OPRD1* in the endogenously expressing DMS-53 cells to that in the engineered HCT-116/ δ OR cells. As expected, DMS-53 cells have significantly lower expression (~60 fold) than HCT-116/ δ OR cells ($p = 0.01$).

As mRNA expression does not necessarily reflect the protein expression levels, we validated the δ OR protein expression in the DMS-53 and H1299 lung cancer cell lines. Initially, we tested to confirm for the presence or absence of δ OR protein in these cell lines. Since the commercially available fluorescence microscope detectors are generally inefficient at 800 nm wavelengths, we used the previously reported Cy5 dye conjugate (Dmt-Tic-Lys-Cy5)^{44, 46} for the fluorescence microscopy studies. Cells were incubated with Dmt-Tic-Lys-Cy5 with or without the presence of excess naloxone, a universal opioid antagonist, as a blocking agent and the fluorescence was imaged by confocal microscopy (Figure 3). As predicted based on the mRNA expression, cell-surface labeling is observed for the DMS-53 cell line while little labeling was observed for the H1299 cell line (Figure 3A). Notably, the cell-surface labeling in the DMS-53 cell line was blocked by naloxone showing the specificity of the labeling, whereas the small amount of labeling in the H1299 cells was unaffected, indicating that binding in these cells was non-specific. Quantification of the fluorescence intensity per cell indicated significantly higher fluorescence for DMS-53 cells labeled with ligand compared to all other conditions ($p = 0.0001$) (Figure 3B).

Next, we sought to determine the receptor number for our endogenous lung cancer cell lines. Previously, our group has used LTRF saturation binding assays to determine receptor number.^{50–52} However, this technique has a lower detection limit of approximately 10,000 receptors/cell. Attempts to quantify the receptor number on the δ OR-expressing DMS-53 cells using this technique were unsuccessful (data not shown). Thus, we conclude that these cells express less than 10,000 receptors. As an alternative to the LTRF saturation binding assay, we used confocal microscopy to semi-quantitatively determine the receptor number. Based on the previously determined K_i for Dmt-Tic-Lys-Cy5, a concentration of fluorescent probe high enough to saturate the receptors was used. The fluorescence of the lung cancer cell lines was compared to the fluorescence of HCT-116/ δ OR cells following labeling with Dmt-Tic-Lys-Cy5. As above, cells were incubated with Dmt-Tic-Lys-Cy5 and fluorescence images were acquired (Figure 4). Cell-surface labeling was observed for the HCT-116/ δ OR and DMS-53 cells (Figure 4A). No labeling was observed for the H1299 cells and the parental HCT-116 cells. The fluorescence intensity per cell was calculated. Based on the results of the quantification, HCT-116/ δ OR cells labeled with ligand had significantly higher fluorescence compared to all other conditions ($p = 0.0001$) (Figure 4B). To approximate the receptor number, we compared the fluorescence of the labeled DMS-53 cells to the fluorescence of the labeled HCT-116/ δ OR cells. As expected, DMS-53 cells have significantly lower fluorescence (~200-fold) than HCT-116/ δ OR cells. The receptor number for HCT-116/ δ OR cells is $1.61 \times 10^6 \pm 1.10 \times 10^5$ δ OR/cell as determined by an *in cyto* time-resolved fluorescence (TRF) saturation binding assay.^{48, 49} Using the fold difference and the receptor number determined by TRF saturation binding assay, we estimate that the δ OR-positive DMS-53 cells contain ~8,000 receptors/cell. It must be emphasized that this number only gives an estimate of the receptor number, as optical data are non-linear.

***In vivo* Tumor Targeting**

Having demonstrated with binding assays, above, that Dmt-Tic-IR800 binds to the δ OR *in vitro*, we were next interested in studying its selectivity *in vivo*. Initial experiments were performed using the HCT-116 parental cell line and HCT-116/ δ OR engineered cells in a bilateral flank subcutaneous xenograft model in mice. Previous experiments using these cell lines and the Dmt-Tic-Lys-Cy5 agent showed selective uptake and retention in the HCT-116/ δ OR tumor at the lowest reliably detectable dose of 4.5 nmol/kg. Due to the longer excitation and emission wavelengths of Dmt-Tic-IR800 in comparison to Dmt-Tic-Lys-Cy5, fluorescence imaging with this probe will not have as much background signal because of lack of tissue autofluorescence. However, we were uncertain as to whether this would result in improved sensitivity at a lower dose. Thus, using the low dose for *in vivo* detection of Dmt-Tic-Lys-Cy5 as a starting point, we selected doses of Dmt-Tic-IR800 that were lower (2.5 nmol/kg), equivalent (5 nmol/kg), and higher (10 and 20 nmol/kg) than the 4.5 nmol/kg used in the prior studies. Mice were injected intravenously with the designated concentrations of Dmt-Tic-IR800 and fluorescence imaging was performed at various time points post-injection using the IVIS-200 imaging system (Figure S5). The data were analyzed for differences in the intensity of the signal with the various concentrations and also for selectivity of the ligand for the target-expressing relative to the control tumors (fold enhancement). From these studies, we determined that 20 nmol/kg and 10 nmol/kg Dmt-Tic-IR800 yielded the greatest tumor-to-background ratio at all time points relative to the other doses and that the dye was retained for days. As the tumor-to-background ratio was similar for the 20 nmol/kg and 10 nmol/kg doses, we selected the 10 nmol/kg dose for further studies so as to minimize the amount of compound used.

Using this optimized dose, we performed another experiment on mice bearing bilateral HCT-116 and HCT-116/ δ OR tumors (Figure S6). We imaged these mice at 24 hours post-injection as this time point showed maximum enhancement differences between the positive and negative tumors. These mice were imaged using the Optix-MX3 fluorescence imaging system. This system uses a pulsed laser, raster scanning illumination and collection, and a time-correlated single photon counting system instead of the epi-illumination and cooled charge coupled device camera found on the IVIS-200. The HCT-116/ δ OR tumors had significantly higher average fluorescence signal than the HCT-116 tumors at 24 hours post-injection of Dmt-Tic-IR800 ($p = 0.01$, $n = 4$) (Figure 5A, 5B, and 5E). We observed a 7-fold enhancement of the positive tumor (HCT-116/ δ OR) relative to the negative tumor (HCT-116). Thus, Dmt-Tic-IR800 is apparently selective for the δ OR *in vivo*.

As the δ OR has significantly higher expression in the engineered cell line compared to the endogenously expressing lung cancer cell lines (see above), we questioned whether Dmt-Tic-IR800 would possess enough sensitivity to detect the lung cancer cell lines *in vivo*. To test this, mice were subcutaneously injected with H1299 (δ OR $-$) and DMS-53 (δ OR $+$) cells in the left and right flanks, respectively. We again performed a dose determination assay to find the optimal concentration of Dmt-Tic-IR800 for further experiments. We hypothesized that we would need a higher concentration of the imaging agent in these experiments due to the lower receptor number. Based on our prior work with fluorescent imaging agents targeting different receptors in endogenous expressing cell lines,⁵³ we selected

concentrations of 40 nmol/kg, 80 nmol/kg, and 160 nmol/kg. Fluorescence imaging was performed using the IVIS-200 imaging system at various time points following intravenous administration of Dmt-Tic-IR800 (Figure S7). For the bilateral flank subcutaneous xenograft model using endogenously expressing cells, the optimal dose was 40 nmol/kg.

Fluorescence imaging of mice bearing H1299 (δ OR $^-$) cells in the left flank and DMS-53 (δ OR $^+$) cells in the right flank was performed using the Optix-MX3 imaging system and a 40 nmol/kg dose of Dmt-Tic-IR800 (Figure S8). At 24 hours post-injection of agent, the DMS-53 (δ OR $^+$) cells had significantly higher normalized intensity than the H1299 (δ OR $^-$) cells ($p = 0.01$, $n = 4$) (Figure 5C, 5D, and 5F). The fold enhancement of the positive tumor (DMS-53) relative to the negative tumor (H1299) was ~4-fold. By the Rose criterion, imaging agents must have a 3-fold enhancement in detection in order to be reliably conspicuous.^{54, 55} Thus, by this criterion, Dmt-Tic-IR800 has sufficient sensitivity to detect endogenously expressing lung cancers and potential for use in clinical settings.

Conclusion

In this work, we have synthesized a near-infrared fluorescent agent Dmt-Tic-IR800. We have demonstrated that this agent retains high binding affinity and specificity for the δ OR both *in vitro* and *in vivo* using a cell line engineered to express the receptor. We have also identified lung cancer cell lines that endogenously express the δ OR and shown that we can image these cell lines in a bilateral flank subcutaneous model.

Currently we are evaluating the pharmacokinetics (PK) and biodistribution (BD) of Dmt-Tic-IR800 in endogenous lung cancer cells. In the future, we will study Dmt-Tic-IR800 using an orthotopic model of lung cancer since this will be a more realistic representation of the clinical problem. We will also test its potential for intraoperative guidance by comparing the use of fluorescence-guidance to traditional white-light surgery. In the clinic, the agent can be used for margin detection during surgery. This capability could improve the rate of complete surgical resection, thereby decreasing the amount of tumor left behind and increasing tumor-free survival. Further, use of this agent to detect sentinel lymph nodes could improve staging of lung cancer, which is important to determine the need for adjuvant chemotherapy without or with adjuvant radiation therapy, and could decrease the morbidity associated with complete lymph node dissection.

Experimental Procedures

Probe Synthesis, Purification, and Characterization

Labeled analog Dmt-Tic IR800, compound **1**, was synthesized using standard Fmoc chemistry on Wang resin as described in Scheme 1 and in detail in Josan, et al.^{44, 46} Briefly, the peptide Dmt-Tic-Lys(Mpr)-OH was assembled on the solid support and cleaved from the resin by treatment with a TFA-scavenger cocktail consisting of trifluoroacetic acid (TFA) (91%), water (3%), triisopropylsilane (3%), and thioanisole (3%) for 3 hours. The peptide was purified by reverse-phase high performance liquid chromatography (RP-HPLC) on a Waters 600 HPLC using a reverse phase C18 column (Vydac C18, 15–20 μ m, 22 $\times$ 250 mm). The peptide intermediate was eluted with a linear gradient of acetonitrile (CH₃CN)/

0.1% TFA (CF₃CO₂H) at a flow rate of 5.0 mL/min. The intermediate was dissolved in dimethyl sulfoxide (DMSO) and treated with 1.3 eq IRDye® 800CW maleimide (Li-cor Biosciences, Lincoln, NE) for 16 hours. The reaction mixture was diluted with water and loaded to the C-18 Sep-Pak™ cartridge (100 mg, Waters, Milford, MA). The cartridge was washed with deionized (DI) water, and then gradually with 5%, 10%, 60%, and 90% aqueous acetonitrile (CH₃CN) to elute the ligand. The purity (>99%) of compound **1** was determined by analytical RP-HPLC using a Waters Alliance 2695 Separation Model with a Waters 2487 dual wavelength detector (220 and 280 nm) on a reverse phase column (Waters Symmetry C18, 3.0 × 75 mm, 3.5 μm). The compound showed an elution time of 13.77 minutes with a linear gradient of 10%-90% aqueous CH₃CN/0.1% CF₃CO₂H at a flow rate of 0.3 mL/min (Figure S1). Electrospray ionization-mass spectrometry (ESI-MS) in negative mode confirmed the structure of compound **1** [(M-2H)²⁻ calc. 852.7667, found 852.4524] (Figure S2).

Characterization of Optical Properties of Dmt-Tic-Lys-Cy5 and Dmt-Tic-IR800

Absorbance and fluorescence excitation and emission spectra were measured with a Tecan Infinite M-1000 PRO multimode microplate reader. Two μL of Dmt-Tic-Lys-Cy5 and Dmt-Tic-IR800 were spotted on each well of a Tecan Nanoquant plate. Absorbance spectra of Dmt-Tic-Lys-Cy5 and Dmt-Tic-IR800 (100 μM in phosphate buffered saline (PBS)) were acquired from 230–1000 nm with a wavelength step size of 2 nm (Figure S3A and S3B). The emission spectrum of Dmt-Tic-Lys-Cy5 (100 μM in PBS) was acquired with an excitation wavelength starting from 230 nm and emission wavelengths from 280–850 nm with an emission wavelength step size of 2 nm and bandwidth of 5 nm (Figure S3C). The emission spectrum of Dmt-Tic-IR800 (1 mM in PBS) was acquired with an excitation wavelength starting from 275 nm and emission wavelengths from 450–850 nm with an emission wavelength step size of 2 nm and bandwidth of 5 nm (Figure S3D).

For the photobleaching experiment, fluorescence intensities of Dmt-Tic-Lys-Cy5 and Dmt-Tic-IR800 were measured following incubation in ambient light or in the dark. Two hundred μL of Dmt-Tic-Lys-Cy5 or Dmt-Tic-IR800 (50 nM in phosphate buffered saline (PBS)) were added to each well (n = 4) of black wall/clear bottom 96 well plates (Corning Costar, Cat. # 3603). Fluorescence images were acquired immediately following addition and at various time points post-addition of the agents using the Xenogen IVIS-200 imaging system (Perkin Elmer, Waltham, MA) equipped with Cy5 (615 to 665 nm excitation/695 to 770 nm emission) and ICG (710 to 760 nm excitation/810 to 875 nm emission) filter sets. Excitation and emission were performed using Cy5 and ICG filter sets for detection of Dmt-Tic-Lys-Cy5 and Dmt-Tic-IR800, respectively. Images were analyzed using Living Image Software (v4.3.1). Image data was analyzed in units of efficiency to enable comparison of the different acquisitions normalized for excitation light levels across the stage. Regions of interest (ROIs) were drawn on the images in the locations of the wells. The percent initial fluorescence intensity was calculated by dividing the fluorescence intensity at the time of interest by the initial fluorescence intensity and multiplying by 100. GraphPad Prism was used to plot the results (Figure S3E). Each data point indicates the average with error bars indicating the standard deviation.

Cell Culture

HCT-116 and DMS-53 cells were obtained from the ATCC (American Type Culture Collection, Manassas, VA). H1299 cells were kindly provided by the Lung SPORE cell line repository at H. Lee Moffitt Cancer Center & Research Institute. HCT-116/ δ OR cells were previously generated in our lab using pcDNA- δ OR15 vector containing a truncated δ OR lacking the final 15 C-terminal amino acids.⁴⁵ HCT-116 and HCT-116/ δ OR cells were cultured in DMEM/F-12 (1:1) media containing 365 mg/L L-Glutamine, 2.438 g/L Sodium Bicarbonate (Life Technologies, Gibco), 10% fetal bovine serum (Atlanta Biologicals), 100 units/mL penicillin, and 100 μ g/mL streptomycin. H1299 cells were cultured in RPMI-1640 media containing 300 mg/L L-Glutamine (Life Technologies, Invitrogen), 10% fetal bovine serum (Atlanta Biologicals), 100 units/mL penicillin, and 100 μ g/mL streptomycin. DMS-53 cells were cultured in RPMI-1640 media containing 300 mg/L L-Glutamine (Life Technologies, Invitrogen) and 10% fetal bovine serum (Atlanta Biologicals). The cells were incubated in 5% CO₂ at 37 °C. Throughout this study, the morphology and growth characteristics of these cells were monitored by microscopy.

Live Cell Binding Assays

To determine binding affinity we used a live-cell lanthanide time-resolved fluorescence (LTRF) competitive binding assay as described previously.^{48, 49} HCT-116 Colorectal cancer cells engineered to express the δ OR (HCT-116/ δ OR) were used to assess ligand binding.⁴⁵ Europium (Eu)-diethylenetriaminepentaacetic acid (DTPA)-[D-Pen², L-Cys⁵] enkephalin (Eu-DTPA-DPLCE), a δ OR agonist, was used as the competed ligand.^{46, 49} To quantify binding affinities by competitive binding assay, the Eu-DTPA-DPLCE binding parameters were determined using the HCT-116/ δ OR cell line by LTRF saturation binding assay as previously described.^{48, 49} The K_d and B_{max} for Eu-DTPA-DPLCE were determined to be 51.76 ± 1.6 nM and $3,011,000 \pm 46,182$ AFU, respectively. For the competitive binding assays, HCT-116/ δ OR cells were plated in black wall/white bottom 96-well plates (Perkin Elmer, Cat. # 6005060) at a density of 20,000 cells per well and were allowed to grow for 3 days. On the day of the experiment, media were aspirated from all wells and then the cells were rinsed with phosphate buffered saline (PBS) (100 μ L/well). Fifty μ L of Dmt-Tic-IR800 (dilutions ranging from 1×10^{-5} to 2.05×10^{-13} M) and 50 μ L of Eu-DTPA labeled DPLCE (10 nM, $K_d = 51.76$ nM) were added to each well. Ligands were diluted in binding assay buffer (Modified Eagles medium [MEM] (Gibco, Cat. # 61100-087), 1 mM 1,10-phenanthroline, 200 mg/L bacitracin, 0.5 mg/L leupeptin, 26 mM NaHCO₃, 25 mM HEPES, 0.2% w/v BSA) and samples were tested in octuplicate. Cells were incubated in the presence of ligands for 1 h at 37 °C and 5% CO₂. Following the incubation, cells were washed three times with wash buffer (50 mM Tris-HCl, 0.2% w/v BSA, 30 mM NaCl) (200 μ L/well). DELFIA enhancement solution (Perkin Elmer, Cat. # 1244-105) was added (100 μ L/well), and plates were incubated for 30 min at room temperature prior to reading. The plates were read on a Perkin Elmer Victor X4 2030 Multilabel Reader using the standard Eu TRF measurement settings (340 nm excitation, 400 μ s delay, and emission collection for 400 μ s at 615 nm). Competition curves were analyzed with GraphPad Prism software using the sigmoidal dose-response (variable slope) classical equation for nonlinear regression analysis. The K_i for Dmt-Tic-IR800 was calculated using the equation $K_i = IC_{50}/(1+[ligand]/K_d)$, where IC_{50} is determined from the competition curves, [ligand] is the final concentration of

Eu-DTPA labeled DPLCE (5 nM) and K_d is the dissociation constant for Eu-DTPA labeled DPLCE (51.76 nM). The number given is the average K_i obtained from three independent experiments.

Determination of δ OR mRNA expression in cell lines using Microarray Data

All selected data were generated using U133 Plus 2.0 arrays from Affymetrix and raw CEL files were available for download. Datasets were identified at the Gene Expression Omnibus at the National Center for Biotechnology Information (NCBI) and ArrayExpress at the European Bioinformatics Institute (EBI) that contained arrays run with lung cancer derived cell lines. The samples used in this analysis were from the accession numbers: GSE5816, GDS2604, GSE5816, GSE4824, GSE7562, GSE8332, GSE10843, GSE13309, GSE14315, GSE14883, GSE15240, GSE16194, GSE17347, GSE18454, GSE21612, and E-MTAB-37.

All CEL files were loaded into the Affymetrix Expression Console software and processed using the MAS 5.0 algorithm to calculate signal intensities using a trimmed mean average of 500 to scale all samples. The quality of individual samples was evaluated from the quality metrics from the Expression Console reports, R QC reports, and an RNA quality analysis tool developed at the Moffitt Cancer Center. Samples were rejected for having high scaling factors (>12), low percent present calls (<35), high RNA quality scores (>4.0), and odd looking scatter plots when compared to a reference array. Data corresponding to the δ OR gene (*OPRD1*), Affymetrix probe 207792_at, were extracted from the full array data to determine the relative expression among the different cell lines (Figure S4).

Determination of δ OR mRNA expression in cell lines using Quantitative Real-Time Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR)

RNA extractions were performed on cell lines using the RNeasy®Mini Kit (Qiagen, Cat. #74104) following the manufacturer's instructions which include the DNase digestion steps. RNA concentration and purity (A_{260}/A_{280} ratio) were determined by using the Nanodrop Spectrophotometer, ND-1000. qRT-PCR was performed using the Smart Cycler (Cepheid, Sunnyvale, CA). *OPRD1* specific primer sets were designed using Gene Runner software for Windows v 3.05: forward, 5'-GGTGACCAAGATCTGCGTGTTTC-3' and reverse, 5'-TTCTCCTTGGAGCCCGACAG-3'. The iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Cat. #170-8893) was used for qRT-PCR. During each experiment, reactions were performed using template without RT mix and with no-template added as controls. β -actin (*ACTB*) was used for normalization.⁵⁶ The following conditions for thermocycling were used: Stage 1 was held at 50 °C for 10 min for cDNA synthesis; stage 2 was held at 95 °C for 5 min for reverse transcriptase inactivation; stage 3 cycled 40 times through two temperatures for PCR amplification, starting with 95 °C for 10 sec and T_m for 30 sec (T_m is 60 °C for *ACTB* and 62 °C for *OPRD1*); and stage 4 included a melt curve for quality control, starting at 40 °C and ending at 95 °C (increasing by 0.2 °C each cycle). Values were calculated as $\text{Expression} = 2^{-Ct(OPRD1)/2^{-Ct(ACTB)}} \times 1000$. Each experiment was repeated 3 times to determine reproducibility.

Determination of δ OR protein expression in lung cancer cell lines by Confocal Microscopy

Confocal microscopy was used to evaluate δ OR protein expression *in vitro*. The expression was evaluated in two lung cancer cell lines, DMS-53 and H1299. δ OR protein expression was determined by labeling the cells with Dmt-Tic-Lys-Cy5. To test non-specific binding, cells were pre-incubated with naloxone (Naloxone hydrochloride dihydrate, Sigma Cat. #N7758), a universal opioid antagonist, prior to the addition of labeled ligand. To evaluate background signal, there were plates of cells that were not incubated with ligand. Cells were grown on glass-bottom plates (World Precision Instrument Fluorodishes 35 mm, Fisher Cat. #50-823-005). DMS-53 cells were plated at a density of 1.5×10^6 cells per plate and H1299 cells were plated at a density of 400,000 cells per plate. The cells were allowed to adhere to the plates overnight and labeling was performed the next day. Media was aspirated from the plates and the cells were rinsed once with PBS. For each of the labeled plates, 2 mL of Dmt-Tic-Lys-Cy5 (50 nM in RPMI-1640 media) was added. Cells were incubated in the presence of ligand for 15 min at room temperature. For each of the blocked plates, 1 mL of naloxone (10 μ M in RPMI-1640 media) was added to the cells for 15 min at room temperature followed by addition of 1 mL of Dmt-Tic-Lys-Cy5 (100 nM in RPMI-1640 media) for 15 min at room temperature. The final concentrations for the blocked plates were 5 μ M naloxone and 50 nM Dmt-Tic-Lys-Cy5. After labeling, the ligand was removed and the cells were rinsed once with PBS and were then fixed with 4% paraformaldehyde (Paraformaldehyde solution 4% in PBS, Affymetrix Cat. #19943) for 10 min. Following fixation, cells were washed two times with PBS containing 50 mM glycine. The background control plates were treated using the same conditions omitting the labeling step. The cells were stored in PBS for imaging. On the day the microscopy was performed, Hoechst 33342 (NucBlue Live Ready Probes, Life Technologies Cat. #R37605) was added to the cells approximately 30 min prior to imaging. Confocal z-stack images at 0.5 nm sections were acquired with a Leica SP5 line scanning confocal microscope using a 63X oil objective, 405 nm diode and 633 nm HeNe laser for excitation and tunable emissions set to spectra for Cy5 and Hoechst 33342, respectively. LASAF v 3.0 software was used to obtain the images and prepare maximum projections from the z-stacks. The experiment was performed in triplicate. Three fields of view were imaged per plate.

Analyses were performed on raw maximum projection images using Definiens Developer v2.0 software. First nuclei were enumerated using an autothreshold segmentation on the Hoechst stain. Then the mean intensity and total pixel area for Cy5 staining was determined in each cell. The total Cy5 signal was determined by multiplying the mean Cy5 intensity by the area of Cy5 staining (Pixel). The total Cy5 signal per cell was determined by dividing the total Cy5 signal by the number of nuclei. The total Cy5 signal per cell was calculated for each field of view. The number of fields of view analyzed were between 8 and 9. GraphPad Prism was used to plot the results. Each data point indicates the average with error bars indicating the standard deviation.

Semi-quantitative Determination of δ OR Receptor Number in lung cancer cell lines

In order to semi-quantitatively determine the receptor number on the lung cancer cell lines, the fluorescence of these cell lines was compared to the fluorescence of HCT-116/ δ OR cells following labeling with Dmt-Tic-Lys-Cy5. HCT-116/ δ OR cells are colorectal cancer cells

engineered to express the δ OR.⁴⁵ The receptor number for HCT-116/ δ OR cells was determined to be $1.61 \times 10^6 \pm 1.10 \times 10^5$ δ OR/cell using an *in cyto* LTRF saturation binding assay as described previously.^{50–52} Confocal microscopy was used to image the δ OR protein expression *in vitro*. The experiment was performed on the DMS-53 and H1299 cells, the two lung cancer cell lines used above, as well as the parental HCT-116 and HCT-116/ δ OR cells. As above, δ OR protein expression was determined by labeling the cells with Dmt-Tic-Lys-Cy5. To evaluate background signal, there were plates of cells that were not incubated with ligand. Cells were grown on glass-bottom plates (World Precision Instrument Fluorodishes 35 mm, Fisher Cat. #50–823-005). DMS-53 cells were plated at a density of 1.5×10^6 cells per plate and H1299, HCT-116, and HCT-116/ δ OR cells were plated at a density of 400,000 cells per plate. The cells were allowed to adhere to the plates overnight and labeling was performed the next day. The background control plates were treated using the same conditions omitting the labeling step. Labeling and imaging of the cells was performed using the protocol described above for characterization of δ OR protein expression. The laser and gain settings on the confocal microscope were reduced for this experiment relative to the experiment above in order to avoid saturation of the fluorescence detector when imaging the HCT-116/ δ OR cells. The experiment was performed in triplicate on HCT-116 and HCT-116/ δ OR cells and in singlicate on DMS-53 and H1299 cells. Three fields of view were imaged per plate.

The images were analyzed as described above for characterization of δ OR protein expression. The number of fields of view analyzed was between 3 and 9. GraphPad Prism was used to plot the results. Each data point indicates the average with error bars indicating the standard deviation.

Tumor Xenograft Studies and Fluorescence Imaging

All procedures were in compliance with the Guide for the Care and Use of Laboratory Animal Resources (1996), National Research Council, and approved by the Institutional Animal Care and Use Committee, University of South Florida. Female nu/nu mice 6–8 weeks old (Harlan Laboratories, Indianapolis, IN) were injected subcutaneously (s.c.) with 8×10^6 HCT-116/ δ OR cells in the right flank and the same number of parental cells in the left flank. Tumors were allowed to grow for 2 weeks. Four days prior to imaging the mice were switched to fluorescence imaging feed (AIN 93G). For the dose determination studies, mice were injected with one of four doses of Dmt-Tic-IR800 (20 nmol/kg, 10 nmol/kg, 5 nmol/kg, or 2.5 nmol/kg) in 100 μ L PBS via the tail vein. Animals were anesthetized using isoflurane (flow 2–2.5 L/min) and were positioned on the heated stage for imaging. Fluorescence images were acquired pre-injection and at various time points post-injection of the ligand using the Xenogen IVIS-200 imaging system (Perkin Elmer, Waltham, MA) equipped with a 710 to 760 nm excitation filter and 810 to 875 nm emission filter (ICG filter set). Animals were kept in a dark chamber between imaging sessions to minimize bleaching of the fluorescent dye. The data were analyzed for differences in signal between the target-expressing and control tumors and for selectivity of the ligand (Figure S5). An optimized dose of 10 nmol/kg Dmt-Tic-IR800 was selected for the following studies. For selectivity studies, tumors were established as described above. Mice ($n = 4$) were injected via the tail vein with 10 nmol/kg Dmt-Tic-IR800 in 100 μ L PBS. *In vivo* fluorescence images were

acquired pre-injection and at 24 hours post-injection of the ligand using the Optix-MX3 (Advanced Research Technologies, Inc., a subsidiary of SoftScan Healthcare Group, Montreal, Canada) (Figure S6). Animals were positioned on a heating pad and anesthetized using isoflurane (flow 2–2.5 L/min). Fluorescence images were acquired using a scan resolution of 1.5 mm and a 785 nm pulsed laser diode with 40 MHz frequency and 12 ns time window. Animals were kept in a dark chamber between imaging sessions to minimize bleaching of the fluorescent dye.

For lung cancer xenografts with endogenous expression levels, female nu/nu mice 6–8 weeks old were injected s.c. with 20×10^6 DMS-53 cells in the right flank and 12×10^6 H1299 cells in the left flank. Tumors were allowed to grow for 3 weeks. Four days prior to imaging the mice were switched to fluorescence imaging feed (AIN 93G). For the dose determination studies, mice were injected with one of three doses of Dmt-Tic-IR800 (160 nmol/kg, 80 nmol/kg, or 40 nmol/kg) in 100 μ L PBS via the tail vein and *in vivo* fluorescence images were acquired as above using the Xenogen IVIS-200 imaging system. The data were analyzed for differences in signal between the target-expressing and non-expressing tumors and for selectivity of the ligand (Figure S7). An optimized dose of 40 nmol/kg Dmt-Tic-IR800 was selected for the following studies. For selectivity studies, tumors were established as described above. Mice ($n = 4$) were injected via the tail vein with 40 nmol/kg Dmt-Tic-IR800 in 100 μ L PBS and *in vivo* fluorescence images were acquired as above using the Optix-MX3 (Figure S8).

For dose determination assays on the Xenogen IVIS-200, images were analyzed using Living Image Software (v3.2). Image data was analyzed in units of efficiency to enable comparison of the different acquisitions normalized for excitation light levels across the stage. Regions of interest (ROIs) were drawn on the images in the locations of the tumors. Autofluorescence background was determined by measuring the mean tumor fluorescence signal prior to injection. This value was subtracted from the fluorescence signal of the same ROI post-injection to obtain mean values for each ROI on the images. Images acquired on the Optix-MX3 were analyzed using Optix-MX3 Optiview software (v 3.01). Regions of interest (ROIs) were drawn on the images in the locations of the tumors. Autofluorescence background was determined by measuring the mean tumor fluorescence signal prior to injection. This value was subtracted from the fluorescence signal of the same ROI post-injection to obtain mean normalized intensity values for each ROI on the images.

Statistics

Data from the competitive binding assay are represented as mean $\pm$ SEM. All other data are represented as mean $\pm$ SD. All statistical analyses were performed with GraphPad Prism v 5.04 or v 6.02. Unpaired Student's *t* test was used to determine the statistical significance of differences between two independent groups of variables. Two way ANOVA followed by Tukey's honestly significant difference (HSD) test was used to determine the statistical significance of differences between multiple groups. For all tests, $p < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

δOR	Delta Opioid Receptor
ACTB	β-actin
BD	biodistribution
BSA	bovine serum albumin
CH₃CN	acetonitrile
CT	computed tomography
DI	deionized
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
Dmt	2',6'-dimethyl-L-tyrosine
DPLCE	[D-Pen ² , L-Cys ⁵] enkephalin
DTPA	diethylenetriaminepentaacetic acid
ESI-MS	electrospray ionization-mass spectrometry
Eu	Europium
FDG	¹⁸ F-fluorodeoxyglucose
Fmoc	9-fluorenylmethoxycarbonyl
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSG	honestly significant difference

ICG	indocyanine green
LTRF	lanthanide time-resolved fluorescence
Lys	lysine
MEM	Modified Eagle medium
Mpr	3-mercaptopropionyl residue
MRI	magnetic resonance imaging
NaHCO₃	sodium bicarbonate
NIR	near-infrared
NIRF	near-infrared fluorescent
PBS	phosphate buffered saline
PET	positron emission tomography
PK	pharmacokinetics
qRT-PCR	quantitative real-time reverse-transcriptase polymerase chain reaction
ROI	region of interest
RP-HPLC	reverse-phase high performance liquid chromatography
s.c.	subcutaneous
SD	standard deviation
SEM	standard error of the mean
SPECT	single-photon emission computed tomography
TFA	CF ₃ CO ₂ H, trifluoroacetic acid
Tic	1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid
TRF	time-resolved fluorescence
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
US	ultrasound

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Figure 1. Dmt-Tic-IR800 binds to the delta opioid receptor (δ OR) *in vitro*

Shown is a representative curve from a competitive binding assay. HCT-116/ δ OR cells were incubated with Eu-DTPA-DPLCE (5×10^{-9} M) and increasing concentrations of Dmt-Tic-IR800 (1.02×10^{-13} to 5×10^{-6} M). Dmt-Tic-IR800 competes with Eu-DTPA-DPLCE for binding to the delta opioid receptor on HCT-116/ δ OR cells. This results in lower signals at higher concentrations of Dmt-Tic-IR800. Data are represented as mean $\pm$ SEM ($n = 8$).

A.**mRNA Microarray Results
for *OPRD1* Expression** **B.****qRT-PCR results for *OPRD1*
Expression** **Figure 2. Expression of the delta opioid receptor gene(*OPRD1*) in cell lines**

A) Normalized expression of *OPRD1* based on Affymetrix microarray data. *OPRD1* expression was analyzed for a panel of lung cancer cell lines and shown are the values for the positive and negative cell lines used in this work. Data are represented as mean $\pm$ SD (n = 3 for DMS-53 and n = 15 for H1299). **, p < 0.01 B) The expression of *OPRD1* was quantified by qRT-PCR and normalized to the expression of β -actin. The engineered cells are HCT-116 colorectal cancer cells that overexpress the delta opioid receptor (δ OR). Also shown are the data for the parental HCT-116 cells that do not express the δ OR. The

endogenous cells are lung cancer cell lines that are positive (DMS-53) or negative (H1299) for *OPRD1*. Data are represented as mean $\pm$ SD (n = 3). **, p < 0.01

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Figure 3. Expression of the delta opioid receptor in lung cancer cell lines

A) Representative fluorescence images from confocal microscopy acquisitions. H1299 (δ OR $^-$) cells (top row) and DMS-53 (δ OR $^+$) cells (bottom row) were incubated with Dmt-Tic-Lys-Cy5 (5×10^{-8} M) with (last column) or without (middle column) the presence of excess Naloxone (5×10^{-6} M). Naloxone competes with Dmt-Tic-Lys-Cy5 for binding to the delta opioid receptor resulting in lower signals for the blocked plates. There were also plates of cells that were not incubated with ligand as background controls (first column). Shown in grayscale is the signal from Dmt-Tic-Lys-Cy5 and shown in blue is signal from Hoechst

33342 nuclear stain. Scale bar = 25 microns. B) Quantification of the total Cy5 signal (mean Cy5 intensity $\times$ area of Cy5 staining) per cell. Data are represented as mean $\pm$ SD (n = 8–9).

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Figure 4. Semi-quantitative determination of the delta opioid receptor number in lung cancer cell lines

A) Representative images from fluorescence imaging experiment. H1299 (δ OR $-$) cells (first column), DMS-53 (δ OR $+$) cells (second column), HCT-116 (δ OR $-$) cells (third column), and HCT-116/ δ OR (δ OR $+$) cells (fourth column) were incubated with Dmt-Tic-Lys-Cy5 (5×10^{-8} M) (bottom row). There were also plates of cells that were not incubated with ligand as background controls (top row). Shown in grayscale is the signal from Dmt-Tic-Lys-Cy5 and shown in blue is signal from Hoechst 33342 nuclear stain. Scale bar = 25 microns. B)

Quantification of the total Cy5 signal (mean Cy5 intensity $\times$ area of Cy5 staining) per cell.
Data are represented as mean $\pm$ SD (n = 3–9).

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Figure 5. Dmt-Tic-IR800 shows selectivity for the delta opioid receptor (δOR) *In vivo*
In vivo fluorescence imaging with Dmt-Tic-IR800 in engineered cells (A, B, and E) and endogenous lung cancer cells (C, D, and F). A and C) Representative fluorescence images acquired pre-injection and 24 h post-administration of Dmt-Tic-IR800 with a lower threshold set to eliminate background signal. A) 10 nmol/kg and C) 40 nmol/kg agent was administered to mice with A) bilateral HCT-116 (δOR-) and HCT-116/δOR (δOR+) and C) bilateral H1299 (δOR-) and DMS-53 (δOR+) tumors in the left and right flanks, respectively. B and D) The figures depict the same 24 h acquisitions shown in A and C),

except with background-subtracted fluorescence signal obtained from regions of interest drawn around the tumors and kidneys. These ROI derived images are adjusted to the same scale. E and F) The graphs depict the mean normalized fluorescence intensities obtained from E) the HCT-116 (δ OR $^-$) and HCT-116/ δ OR (δ OR $^+$) tumors and F) the H1299 (δ OR $^-$) and DMS-53 (δ OR $^+$) tumors. Data are represented as mean $\pm$ SD (n = 4). **, p < 0.01

Scheme 1. Synthetic Route for Compound 1 (Dmt-Tic-IR800)

ⁱ Solid phase synthesis as described in Josan, et al^{44,46}; ⁱⁱ TFA-scavenger cocktail (TFA (91%), water (3%), triisopropylsilane (3%), and thioanisole (3%)) for 3 h; ⁱⁱⁱ 1.3 equiv IRDye ® 800CW maleimide in DMSO for 16 h.