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Tumor Targeting, Pharmacokinetics and Biodistribution of a Near Infrared Fluorescent-labeled δ -Opioid Receptor Antagonist Agent, Dmt-Tic-Cy5

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Abstract

Fluorescence molecular imaging can be employed for the development of novel cancer targeting agents. Herein, we investigated the pharmacokinetics (PK), biodistribution (BD) and cellular uptake of Dmt-Tic-Cy5, a delta-opioid receptor (δ OR) antagonist-fluorescent dye conjugate, as a tumor-targeting molecular imaging agent. δ OR expression is observed normally in the CNS, and pathologically in some tumors, including lung liver and breast cancers. In vitro, in vivo and ex vivo experiments were conducted to image and quantify the fluorescence signal associated with Dmt-Tic-Cy5 over time using in vitro and intravital fluorescence microscopy and small animal fluorescence imaging of tumor-bearing mice. We observed specific retention of Dmt-Tic-Cy5 in tumors with maximum uptake in δ OR-expressing positive tumors at 3 h and observable persistence for >96 h; clearance from δ OR non-expressing negative tumors by 6 h; and systemic clearance from normal organs by 24 h. Live-cell and intravital fluorescence microscopy demonstrated that Dmt-Tic-Cy5 had sustained cell-surface binding lasting at least 24 h with gradual internalization

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

over the initial 6 h following administration. Dmt-Tic-Cy5 is a δ OR-targeted agent that exhibits long lasting and specific signal in δ OR-expressing tumors, is rapidly cleared from systemic circulation and is not retained in non- δ OR expressing tissues. Hence, Dmt-Tic-Cy5 has potential as a fluorescent tumor imaging agent.

TOC Graphic

Keywords

Fluorescence Imaging Agent; Tumor Targeting; Delta Opioid Receptor Antagonist; Pharmacokinetics; Biodistribution; Dorsal Window Chamber; Cancer Imaging; Intravital Microscopy; Live Cell Imaging; Cancer; Uptake

INTRODUCTION

Non-invasive fluorescence imaging is becoming an important tool in biomedical research for the evaluation of targeting scaffolds that can be used in the development of cancer targeted diagnostic imaging and therapeutic agents. The recent availability of clinical real-time fluorescence imaging systems has opened the door for the development of cancer targeted fluorescent agents for intraoperative use in margin detection and identification of lymph node involvement. Fluorescence imaging of cancer requires the development of imaging agents that selectively bind to cell surface receptors with high expression on cancer cells and minimal expression on normal non-neoplastic tissue. The growing list of cancer-specific receptors identified includes: epidermal growth factor^{1–3}, folate receptor^{4–6}, integrins^{7, 8}, and somatostatin receptors^{9–13}. Currently there are a variety of fluorescent-labeled cancer imaging agents either in development or commercially available that target the receptors listed above.^{14–20} The labeling of tumors in vivo by novel cancer targeted peptide agents conjugated with near infrared fluorescent cyanine dyes, like Cy5, has been shown by our group and others.^{21–28}

Despite the current wide array of ligands that bind remarkably well to opioid receptors, the development and evaluation of novel fluorescently labeled opioid receptor agonists or antagonists is limited. The δ -opioid receptor (δ OR) is less well characterized than the other two opioid receptor types (κ - and μ -) that all belong to the G-protein-coupled receptor (GPCR) superfamily.²⁹ Typically, δ OR internalization is agonist-mediated and can occur through G-protein-dependent and G-protein-independent pathways, that involve

phosphorylation, β -arrestin, or clathrin-coated vesicles.³⁰ No internalization of δ ORs has been observed with antagonists. In 2012, Granier et al. reported the crystal structure of murine δ OR bound to naltrindole³¹, one of the few highly potent and δ -selective antagonists (e.g. naltriben^{32, 33}, naltrindole^{32, 34, 35} and Dmt-Tic^{36–39}).

The δ OR has been reported to be expressed in various human cancers, such as lung, liver, and breast.^{40–43} Even though the full mechanism is not yet known, δ OR has a role in driving tumor progression.^{41, 44–46} Recent studies suggest that inhibition of δ OR decreases cellular proliferation leading to apoptosis of tumor cells.^{44, 47, 48} Therefore, using a fluorescently-labeled δ OR antagonist for targeting may be preferred over an agonist because they may have anti-proliferative/anti-tumor effects and will likely have a higher, longer lasting fluorescence signal due to slower off rates when binding to the receptor⁴⁹ and decreased potential for quenching of the fluorescent dye in the low pH environment of the lysosome. To the best of our knowledge, our Dmt-Tic-Cy5 is the only account of a fluorescent δ OR antagonist targeted peptide agent that exhibits high binding affinity ($K_i = 3$ nM).²¹

Herein, we investigate the potential of using Dmt-Tic-Cy5 as a tumor targeted fluorescent-labeled molecular agent by characterizing cellular uptake, tumor targeting, pharmacokinetics (PK) and biodistribution (BD) of this agent in vivo using mouse tumor models with and without expression of the δ OR and acquisition of fluorescence images over time following intravenous (i.v.) administration.

EXPERIMENTAL SECTION

Fluorescent δ OR-targeted Agent Synthesis

Dmt-Tic-Cy5 (Dmt-Tic-Lys(Cy5)-OH), a potent ($K_i = 3$ nM) δ -opioid receptor antagonist conjugated to Cy5 fluorescent dye, was synthesized by solid-phase synthesis as previously described.²¹

Cell Culture

The parental HCT 116 (ATCC #CCL-247) cell line was purchased from ATCC (Manassas, VA). The HCT 116/ δ OR colon cancer cells were genetically engineered to highly over-express the δ -opioid receptor.^{50, 51} Expression of the δ OR on the surface of HCT 116 and HCT 116/ δ OR cells was characterized prior to injection using an in vitro time-resolved fluorescence (TRF) binding assay.²¹ For the HCT 116/RFP cells, parental HCT 116 cells were engineered to express RFP (red fluorescent protein) and were used as the negative, non- δ OR expressing tumor control for the intravital imaging experiments.⁵² Upon completion of experiments, cell lines were authenticated using short tandem repeat (STR) DNA typing according to ATCC's guidelines.⁵³ The cell lines were cultured in DMEM/F12 media (Invitrogen, Carlsbad, CA) supplemented with 10% normal calf serum (Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin/streptomycin solution (Sigma, St. Louis, MO).

mRNA Expression Using Quantitative Real time RT PCR (qRT-PCR)

The relative mRNA expression levels of OPRD1, the gene that encodes the δ OR, were determined using quantitative real-time RT-PCR (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 (A260/A280 ratio) were determined by using the Nanodrop Spectrophotometer, ND-1000 (Wilmington, DE). 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 without template added as controls. β -actin (ACTB) was used for normalization.⁵⁴ 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 s and T_m for 30 s (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 $\text{Expression} = 2^{-\text{Ct(OPRD1)}/2^{-\text{Ct(ACTB)}}} \times 1000$. Expression of OPRD1 was normalized to the expression of β -actin in each cell line. Each experiment was performed in triplicate.

Receptor Number Determination

To verify the expression of δ OR on HCT 116/ δ OR cells and no expression on the HCT 116 parental cell line, TRF saturation binding assays were performed as previously described.^{51, 55–57} The number of δ -opioid receptors expressed on the cell surface of HCT 116/ δ OR cells was calculated using an adapted version of the binding assay, as previously described.⁵⁶ Each data point indicates the average of four assays with 4 replicates, with error bars indicating the standard error of the mean. To calculate the number of receptors per cell, standard curves of the relationship between fluorescence intensities and ligand concentrations were generated. The standard curves were then used to determine the amount of ligand present at the B_{max} obtained in the saturation binding assay. The average number of cells per well at the end of the assay was calculated. To determine the receptor number, the following equation was used: $(\text{Eu amount for } B_{\text{max}} (\text{mole})/\text{avg. cell number per well}) \times 6.023 \times 10^{23} = \text{receptor number per cell}$.

In Vitro Live Cell Epifluorescence Microscopy

Live cell epifluorescence microscopy was used to evaluate cellular uptake of the Dmt-Tic-Cy5 agent in vitro. HCT 116/ δ OR cells were grown on glass-bottom plates (World Precision Instrument Fluorodishes 35 mm). Cellular internalization of the Dmt-Tic-Cy5 agent was monitored by acquiring fluorescence images from 0 to 24 h with a Zeiss Z1 observer microscope (Carl Zeiss Inc., Germany) using a 40 $\times$ oil objective, Cy5 filter cube, and a MRm3 CCD camera with an exposure time of 1.5 s. The microscope is equipped with a fully enclosed incubation chamber set to 37°C and 5% CO₂. Axiovision v4.8.2 software was

used to obtain the images and the time-lapse sequences. The cells were rinsed once with PBS and Dmt-Tic-Cy5 (2.5 nM in Fluorobrite DMEM media) was added. Immediately following ligand addition, the plate was placed on the stage in the incubation chamber and images were acquired at 30 s intervals for 15 min. After 15 min, the media was removed and the cells were rinsed once with Fluorobrite DMEM media. The cells were incubated in Fluorobrite DMEM media for the remainder of the imaging session. The mark and find feature was used to image 4 random fields of view every 10 min for 24 h starting approximately 30 min post-ligand addition. The experiment was performed in triplicate on consecutive days.

Axiovision v4.8.2 software was used to analyze the images. To determine cell uptake rates, the fluorescence was measured in the cytoplasm (inside) and on the cell membrane (outside) by drawing regions of interest (ROIs) on the images from various time points. The cells were tracked throughout the image sequence. For quantification, a ROI was drawn outside the cell membrane to measure the total fluorescence for the whole cell. Another ROI was drawn immediately inside the cell membrane to measure the fluorescence for the cytoplasm (inside).

For each region, the fluorescence was determined by multiplying the average fluorescence intensity by the area. The fluorescence on the cell membrane (outside) was determined by subtracting the fluorescence of the cytoplasm (inside) from the total fluorescence. The difference of fluorescence for the cytoplasm (inside) to fluorescence on the cell membrane (outside) was calculated for each cell at each time point. The ratio inside/outside for individual cells was averaged for each day at each time point. The number of cells analyzed per day was between 11 and 25. GraphPad Prism 6 was used to plot the results. Each data point indicates the average of three days, with error bars indicating the standard deviation.

Animals

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 under the approved protocols R3715 and R4002. Immunocompromised mice were housed in a clean facility with special conditions that include HEPA filtered ventilated cage systems, autoclaved bedding, autoclaved housing, autoclaved water, irradiated food and special cage changing procedures. Mice were handled under aseptic conditions including the wearing of gloves, gowns and shoe coverings.

Dose Determination for In Vivo Imaging Studies

Cells (8×10^6) were xenografted into the left (HCT 116) and right (HCT 116/8OR) flanks of female nu/nu mice (Harlan, Indianapolis, IN). After 1 week, tumors were ready for imaging. A range of dosages from 0.005 to 140 nmol/kg of Dmt-Tic-Cy5 were administered via tail i.v. injection. Animals were imaged immediately to check for successful injection. Follow-up imaging was performed 24 h post-injection. Imaging was performed using an IVIS Imaging System, 200 Series (PerkinElmer Xenogen Caliper Life Sciences, Hopkinton, MA). Excitation (615–665 nm) and emission (695–770 nm) filters were used in wavelength ranges

suitable for in vivo excitation and detection of emitted light of Cy5 dye. A second measurement using a blue shifted excitation filter set (580–610 nm) was used to determine autofluorescence. The ratio of the two measurements was used as a correction factor (k) to subtract background signal (B) from Cy5 fluorescence signal (A) using the equation: $A - B * k$. Instrument background was determined by removing the animals and repeating the measurements. Instrument background was subtracted prior to autofluorescence background subtraction. Acquisition times ranged from 4 s to 10 s in order to keep intensity counts above a minimum of 15,000 but below saturation values of ~60,000. Results are displayed in efficiency units in which the fluorescence surface radiance values (photons/sec/cm²/sr) within an image are normalized using a stored reference image that represents the variation in excitation light intensities across the stage, so that images acquired at different times and locations on the stage can be directly compared. The autofluorescence ratio was determined by drawing identical ROIs on the instrument background subtracted Cy5 fluorescence image and corresponding autofluorescence image, and measuring radiance values for both ROIs.

Pharmacokinetics

We chose to evaluate the pharmacokinetics (PK) of the Dmt-Tic-Cy5 agent in vivo over a time-course (0–168 h) after the administration of a single dose at a final concentration of 4.5 nmol/kg. δ OR– and δ OR+ engineered HCT 116 cells (8×10^6) were bilaterally xenografted into left and right flanks of athymic nude female mice, respectively. Tumor volume was determined with calipers using the formula: volume = (length $\times$ width²)/2. Mice were ready for fluorescence imaging after reaching an average size of 500 mm³. Mice were imaged in vivo over the time course (3 min, 9 min, 15 min, 30 min, 45 min, 1.5 h, 3 h, 6 h, 12 h, 18 h, 24 h, 48 h, 72 h, 96 h, 168 h) using the IVIS 200 system and Cy5 filter sets. The data were analyzed after performing instrument background subtractions and tissue autofluorescence corrections. Autofluorescence was determined using a pre-injection acquisition. The fluorescence signals were quantified using Living Image Software. ROIs were drawn and mean surface radiance normalized for heterogeneity of stage lighting (efficiency units) was used. One-point binding assays were done to demonstrate that the HCT 116 cells did not express δ OR and the HCT 116/ δ OR cells did express δ OR prior to the implantation of the cells into the animals.

Biodistribution Studies

To confirm the selectivity of the Dmt-Tic-Cy5 agent in the PK studies, both in vivo and ex vivo fluorescence imaging acquisitions were performed on an additional group of mice to determine the biodistribution (BD) of Dmt-Tic-Cy5. At select time points (0, 7.5 min, 15 min, 45 min, 3 h, 6 h, 24 h, 48 h) post administration of 4.5 nmol/kg Dmt-Tic-Cy5 agent, mice were in vivo fluorescence imaged, euthanized, tumors and organs (spleen, pancreas, liver, kidneys, GI tract, heart, lungs and tumors) harvested and imaged ex vivo to determine the agent's BD. Imaging at only a single time-point per animal is useful to determine if photobleaching of the Cy5 dye altered the observed fluorescence signal during the multiple acquisition time courses in the PK study above. The data were analyzed in the same manner as in the PK study described above.

Intravital Tumor Cell Uptake Studies

Intravital confocal microscopy was used to evaluate agent circulation, extravasation and cellular uptake of the Dmt-Tic-Cy5 agent in vivo using our previously described mouse dorsal skinfold window chamber (DWC) model (Supporting Figure S2).^{52, 58, 59}

Cellular internalization of the Dmt-Tic-Cy5 agent was monitored from 0 to 24 h using confocal fluorescence microscopy with a 25× lens and an acquisition rate of 3570 pixels/min. Initially, images were continuously acquired for 30 min post-injection of 45 nmol/kg Dmt-Tic-Cy5 agent via tail vein and at several other time points up to 24 h. To determine extravasation rates, the mean fluorescence intensity was measured in the vessel interior and in the tumor tissue adjacent to the vessel by drawing ROIs on the confocal image sets from various time points using ImagePro v6.2. To determine cell uptake rates of agent, the average intensity of each image was determined using Definiens Developer v2.0. The units of intensity are the dynamic range value 0–255 for an 8 bit image, where 0 is black and 255 is pure white. For presentation purposes, the greyscale agent-related fluorescence signal is depicted in red by pseudo color.

Statistics

Data are represented as mean $\pm$ s.d. and Student's t-test was used to determine statistical significance.

RESULTS

Receptor Number Determination

For this study, HCT 116 colon carcinoma cells engineered to express the δ OR (HCT 116/ δ OR) were used as a target receptor positive in vitro cellular and in vivo tumor xenograft model and the parental HCT 116 cells were used as the target negative control.²¹ To characterize δ OR expression levels in these cell lines, quantitative real-time RT-PCR (qRT-PCR) was used to determine the relative mRNA expression levels of OPRD1, the gene that encodes the δ OR. The expression of OPRD1 was negligible in the HCT 116 parental cells and was significantly higher in the HCT 116/ δ OR cells (p-value <0.05, n = 3) (Figure 1A). As another measure of δ OR expression, a one-point saturation binding study using the high affinity europium-labeled δ OR ligand, Eu-DPLCE⁵¹ and our previously described TRF assay⁵¹, demonstrated high expression of δ OR on the HCT 116/ δ OR cell line with low nonspecific binding (p-value <0.0001, n=4), and there was no significant difference in total binding compared to nonspecific binding on the HCT 116 parental cells (p-value >0.05, n=4) (Figure 1B). These results indicate that δ OR is either not expressed on the surface of the parental HCT 116 cells or that it is expressed only at low levels that cannot be detected above background signal. To determine the target receptor number expressed on the cell surface of the HCT 116/ δ OR cells, a saturation binding assay was performed using Eu-DPLCE.⁵¹ The specific binding curve for δ OR (total minus nonspecific) (Figure 1C) was used to calculate the Eu-DPLCE K_d of 51.76 ± 1.6 nM and B_{max} of $3,011,000 \pm 46,182$ AFU ($R^2 = 0.999$, n=4). The average numbers of HCT 116/ δ OR cells per well were $76,700 + 5,430$ (n=6). The B_{max} value was determined to be $205 + 5.13$ fmol per well (Figure 1D). The amount of δ OR expressed on the cell surface was thus calculated to be 1,610,000

+ 110,100 on this stably expressing cell line. We have previously reported the K_d and B_{max} for Eu-DPLCE as 15.3 nM and 770,000 respectively using CHO/ δ OR cells.⁵¹ The minor difference observed in the K_d determination using HCT 116/ δ OR cells relative to the CHO/ δ OR cells is possibly due to differences in the chemical and ionic environments on the surfaces of the two different cell lines.

In Vitro Microscopic Observation of Cell surface Labeling and Live Cellular Uptake

Using digital imaging epifluorescence live-cell microscopy, we evaluated the in vitro live cell labeling of the HCT 116/ δ OR cells with Dmt-Tic-Cy5. Figure 2A shows representative images of cells over time from 30 min to 1030 min (~17 h) following addition of 2.5 nM Dmt-Tic-Cy5. High cell-surface labeling was observed ~2 min after addition of ligand, the time it took to set-up and begin acquiring images, and some fluorescence was detected on the cell-surface for the entire 24 h. Cellular uptake of Dmt-Tic-Cy5 over the time course was observed, and was consistent with internalization of the fluorescence. Figure 2B shows that the fluorescence was gradually internalized over time from 30 to 430 min (~7 h) as indicated by increasing amounts of fluorescence in the cytoplasm (inside) relative to the cell membrane (outside) with a maximum value of 1.69 for the ratio of inside/outside. Once established, this fluorescence ratio remained constant up to 24 h post-ligand addition.

Dose Determination for In vivo Fluorescence Imaging

To determine an optimal dose of Dmt-Tic-Cy5 for studying PK and BD in vivo via fluorescence imaging, we initially tested dosages ranging from 0.01 nmol/kg to 200 nmol/kg by quantifying in vivo tumor fluorescence at 24 h post-intravenous administration. The highest fold of enhancement (FOE, 5 fold) of fluorescence signal in the δ OR+ tumors relative to the δ OR– tumors was observed at the 50 nmol/kg dosage 24 h post-administration (Supporting Figure S1A & S1C), and this was also the highest dosage where the negative tumor had completely cleared of agent by 24 h. However, since photobleaching of the agent had been observed during in vitro studies, we discovered that by housing the animals in a dark chamber post-injection and between imaging time-points, a lower dosage of 4.5 nmol/kg generated comparable fluorescence signal and increased the FOE to 22 at 24 h (Supporting Figures S1B & S1D). In contrast to the dark chamber results, animals housed in diurnal lighting had no residual fluorescence at 24 h post-administration of dosages up to 50 nmol/kg. Based on the Rose criterion, a threshold signal-to-noise ratio of 3 to 5 is required to reliably detect differences in image features.⁶⁰ Hence, the ~20 FOE observed using the 4.5 nmol/kg dosage after housing in a dark chamber was sufficient and this dosage was selected for use in the PK and BD studies.

Pharmacokinetics

The PK of Dmt-Tic-Cy5 was evaluated in a δ OR+/ δ OR– bilateral subcutaneous tumor xenograft animal model, in which a series of fluorescence images were acquired over a time course (0–168 h) (Figure 3). Differential uptake of the agent, i.e. elevated fluorescence in the δ OR+ tumor relative to the δ OR– tumor, was first observed at 3 min post-injection with the fluorescence signal increasing to a maximum in the positive tumor at 3 h and was still visible in the positive tumor 96 h post-administration (Figure 3B). Fluorescence in the negative tumor increased to a maximum at 15 min and was reduced to nearly pre-injection

levels by 6 h post-administration (Figure 3B). Enhancement in the positive tumor relative to the negative tumor was significantly higher from 3 min to 96 h, $p < 0.03$ and >3 -fold higher from 30 min to 96 h post-administration (Table 1). High enhancement, ~ 18 -fold was observed as early as 6 h and remained high until 24 h (~ 21 fold). After 168 h, there were no significant levels of the Dmt-Tic-Cy5 related fluorescence detected in the mouse or tumor. The presence of Dmt-Tic-Cy5 in other organs (kidneys and liver) was also observed in vivo for up to 24 h (Figure 3A). By plotting the range of values with linear increases and decreases in fluorescence for both positive and negative tumors and analyzing by linear regression line fits, the slopes were used to estimate the rates of uptake and clearance (Figure 3C, Table 2).

Biodistribution

An ex vivo BD study using fluorescence imaging at select time points confirmed the uptake, selectivity and clearance of Dmt-Tic-Cy5 in the bilateral tumor xenograft animal model. Figure 4 shows agent related fluorescence signal in the excised tumors, kidneys and liver at time-points ranging from 0 to 48 h post-administration. The uptake in the $\delta\text{OR}+$ tumor was significantly elevated relative to the $\delta\text{OR}-$ tumor from 7.5 min to 48 h ($n=5$, p -value < 0.05) (Figure 4B). The highest fluorescence signal in both the kidney and liver occurred at 15 min and steadily decreased to pre-injection levels by 24 h post administration (Figure 4C). At 15 min post-administration, the signal in the liver was higher than any of the other tissues, including the $\delta\text{OR}+$ tumor. After 24 h, the fluorescence had nearly cleared from all organs and tissues except for the $\delta\text{OR}+$ tumor, demonstrating selectivity. There was minimal to no fluorescence signal associated with uptake of the Dmt-Tic-Cy5 agent observed in the heart, lungs, GI tract, spleen and pancreas (Supporting Figure S3). The quantified Dmt-Tic-Cy5 related fluorescence values observed in the tumor xenografts and organs in this ex vivo biodistribution study were comparable to results observed in the in vivo PK study.

Intravital Tumor Cell Uptake Studies

We evaluated the kinetics of agent circulation, extravasation, and tumor and cellular uptake and clearance of the Dmt-Tic-Cy5 agent using a dorsal skin-fold window chamber (DWC) xenograft tumor model and time-lapse intravital confocal microscopy (Supporting Figure S2). Following the establishment of target receptor positive (HCT 116/ δOR) or negative (HCT 116/RFP) tumors and patent vasculature in the DWC model, vascular circulation time and rates of extravasation were determined by confocal fluorescence acquisitions at 1, 1.5, 2 and 5 min following administration of Dmt-Tic-Cy5. Regions of interest (ROI) were used to measure agent related fluorescence within a δOR negative tumor vessel and immediately outside the vessel within the surrounding tumor tissue (Figure 5A). The Dmt-Tic-Cy5 agent reached peak intensity by 1 min post-injection and was cleared from the tumor vasculature by 2 min post injection (5B). As a measure of extravasation, fluorescence increased at a linear rate immediately outside the vessel in the surrounding tumor tissue for the first 2 min and was further increased at the final 5 min acquisition (Figure 5C).

Uptake and clearance for both δOR positive and negative tumors were determined by quantifying the increase and decrease in fluorescence intensity in a tumor ROI over time (Figure 6A & B). Agent related fluorescence intensity was highest in the negative tumor at

10 min post-administration and in the positive tumor at 30 min. By 24 h, fluorescence was cleared from the negative tumor; while the positive tumor still exhibited a strong fluorescence signal. By 30 minutes post-administration, fluorescence was observed in the positive tumor around the edges of the cells and by 24 h, fluorescence was observed inside the positive tumor cells, indicating cellular uptake.

DISCUSSION

By in vitro, in vivo, ex vivo and intravital fluorescence imaging, we have characterized the pharmacokinetic and biodistribution properties of Dmt-Tic-Cy5, using fluorescence as a surrogate for compound concentration. Compound concentration can be underestimated by this method in the context of photobleaching or acid-quenching of the fluorochrome in low pH conditions, e.g. in the lysosome after internalization. Our results demonstrated that for the Dmt-Tic-Cy5 compound, fluorescence levels can be decreased 5 to 6 fold at 24 h post-administration by housing nude mice in diurnal lighting as opposed to dark chamber (Supporting Figure S1). In order to minimize this effect, agent was divided into aliquots after resuspension and kept in the -80°C freezer in the dark until administration to animals and all animals used in these studies were housed in a dark chamber and kept in low light conditions during imaging sessions after administration of fluorescent agent. Cellular internalization was observed in vivo by 24 h post-administration (Figure 6) and elevated tumor fluorescence was observed out to 96 h post-administration (Figure 3), but we do not know the specific mechanism for decreased fluorescence over time, i.e. compound degradation and secretion, or acid-bleaching of the fluorochrome. However, in another study involving a heterobivalent agonist targeting agent, we have reported the relatively rapid loss of Cy5 fluorescence from the positive tumor, in <24 h, compared to the Eu-DTPA conjugated derivative that was still present at high levels at 48 h, suggesting degradation of the Cy5 fluorochrome but not the targeting scaffold.²⁸ Since tumor fluorescence in the current study lasts many days longer than the heterobivalent agent, this suggests that intracellular fluorochrome degradation, e.g. by acid-quenching, may be less of a factor for the Dmt-Tic-Cy5 agent. This could be due to differential routing of the agent following internalization, i.e. to a compartment other than the lysosome.

Following intravenous administration of 4.5 nmol/kg of agent into a bilateral tumor xenograft model for in vivo PK studies (Figure 3), peak fluorescence was observed after 15 min in the δOR negative tumor with clearance from the tumor by 6 h. The positive tumor accumulated fluorescence to a peak at the 3 h time point, which was the last time point showing some fluorescence in the negative tumor. Hence, the rate of fluorescence uptake was higher in positive tumors relative to negative tumors (Table 2). Intravital tumor imaging shows a similar trend of fluorescence accumulation in the positive tumor beyond the maxima observed in the negative tumor and retention in the positive tumor after complete clearance from the negative tumor (Figure 6). These data suggest that the positive tumor accumulation of the agent continued until the agent was no longer circulating, which occurred sometime between the 3 h and 6 h imaging time point based on the timing of clearance of fluorescence from the negative tumor. Inversely, the positive tumors had a much slower rate of clearance of the fluorescence relative to negative tumors (Table 2), suggesting that negative tumor clearance is associated with systemic clearance, while positive tumor clearance is related to

intracellular degradation and secretion of the compound, or acid-quenching of the fluorochrome.

An important question in the development of targeted agents for delivery of imaging or therapeutic payloads is whether or not, and by what mechanism the agent is internalized following binding to the cell surface. We have demonstrated that Dmt-Tic-Cy5 has high binding affinity and high antagonist activity and the cell line used for the study was engineered to express δ OR with a C-terminal truncation that disrupts intracellular signaling²¹ (Figure 1). Therefore, we anticipated high cell-surface labeling without receptor mediated endocytosis, which occurs within minutes following agonist stimulation of δ OR.⁴⁶ Indeed, live-cell labeling studies using Dmt-Tic-Cy5 demonstrated robust cell-surface labeling, with a gradual rate of internalization 7 h post-addition of agent to positive tumor cells (Figure 2) and a large percentage of the fluorescence signal still being observed on the cell membrane 24 h post-administration. Intravital imaging results were in agreement with the in vitro results, in that cell-surface binding of Dmt-Tic-Cy5 to receptor positive tumor cells was apparent up to 30 minutes post-administration with internalization observed at 24 h (Figure 6). Antagonist-induced internalization of the δ OR has not been observed previously, but agonist-induced δ OR-mediated internalization occurs within minutes.⁴⁶ Antagonist-induced receptor-mediated internalization has been observed for other GPCRs^{61–63}, however the timing of internalization is comparable to agonist-induced, i.e. within minutes, albeit in the absence of intracellular signaling. Hence, the gradual prolonged uptake of our agent suggests a passive, non-receptor mediated, mechanism of uptake possibly associated with membrane recycling or pinocytosis.^{64, 65} For example, although not specifically targeted to a given nascent endosome, any agent-bound receptor that happens to be within an endocytotic, phagocytotic or pinocytotic invagination as it develops in the plasma membrane would be internalized. Hence, we propose that, for the model system used in this study, our agent may be internalized by a gradual process related to the statistical probability that a given bound receptor is located within endosomal membranes as they form. In addition to evaluation of tumor cellular uptake, intravital imaging allowed for evaluation of the timing of tumor vascular circulation and extravasation of the fluorescent molecular imaging agent (Figure 5). Due to low sampling number, the data presented herein cannot be analyzed with statistical rigor. However, these data provide examples of the potential power of intravital imaging in the in vivo characterization of targeted agents at the microscopic scale. Future experiments will involve multiple animals, continuous acquisitions that begin pre administration and continue post-administration, and more frequent time-points in order to better quantify the agent properties.

During the in vivo imaging time-course, fluorescence was also observed to accumulate in the general areas overlaying the kidneys and liver, implicating both as routes of clearance for this agent. Ex vivo fluorescence imaging of the kidneys and liver confirmed these clearance routes, with both organs having peak accumulation at 15 minutes post-administration of agent, which corresponded to the peak accumulation of fluorescence in the negative tumor, and nearly complete clearance by the 24 h time-point (Figure 4). No other organs had retention of the agent over the ex vivo time course, including the spleen, implying that macrophage clearance is not a factor for this agent. Ex vivo tumor uptake and clearance profiles were in agreement with the in vivo imaging data.

At the time of our initial development of this agent²¹, dyes such as Cy5 were considered to have favorable properties for optical imaging with targeted agents. However, we found that the Cy5 dye used for conjugation to the targeting ligand was not an ideal fluorescent dye due to photobleaching of the dye and tissue autofluorescence during imaging. Autofluorescence is the emission of light by biological structures following absorption at a lower wavelength.⁶⁶ During the course of this study, we observed that Dmt-Tic-Cy5 had significantly decreased fluorescence intensity following exposure to light following injection, i.e. in animals kept in caging with diurnal lighting relative to animals kept in a dark chamber after agent administration, indicating photobleaching. In vivo imaging using 615–665 nm excitation light produced significant autofluorescence in the emission wavelengths of 695–770 nm used, which lead to decreased sensitivity. NIR fluorescent dyes with longer excitation and emission wavelengths (700–900 nm), e.g. indocyanine green (ICG) derivatives, have decreased tissue autofluorescence enabling higher signal-to-noise ratios, making them more suitable for in vivo imaging.⁶⁷ Because of this issue, and because ICG dye is approved for clinical use, clinical fluorescence imaging instrumentation, including the Firefly robotic surgical system by Intuitive, the SPY system by Novadaq, the Fluobeam handheld system by Fluoptics, and the SurgVision imaging platform, etc. have been developed for detection of ICG dye and its derivatives. Hence, for future studies using the Dmt-Tic targeting agent, we will conjugate longer wavelength NIR dyes that are compatible with the current clinical instrumentation, are more stable and have higher signal-to-noise ratios due to decreased autofluorescence.

In addition to using Dmt Tic as a targeting scaffold for delivery of fluorescence, it also has potential in development of a δ OR-targeted PET imaging agent. Three-fold enhancement was observed in the positive tumor relative to the negative tumor as early as 3 min post-administration, and near twenty-fold enhancement was seen as early as 6 h remaining high through 24 h. These data indicate the feasibility of using the Dmt-Tic antagonist ligand for development of a PET imaging agent which requires at least three-fold enhancement to distinguish differences in imaging features according to the Rose criterion^{44–46}, and must occur minutes after injection of agent due to the short decay half-lives of PET radioisotopes.

Using agent related fluorescence as a surrogate for concentration, we have further characterized the PK, BD and tumor selectivity of our Dmt-Tic-Cy5 molecular imaging agent. Agent circulation time, extravasation from tumor vasculature, kinetics of tumor and systemic uptake and clearance, target expressing tumor cell uptake and specificity, and biodistribution were evaluated using microscopic and macroscopic fluorescence image acquisitions and in vitro, in vivo and ex vivo tumor models. Greater than 3-fold enhancement was observed in target positive tumors by 3 min, and a high ratio of enhancement, approximately 20 fold, was maintained from 6 h to 24 h post-administration. Systemic clearance was observed by 6 h and clearance from kidneys and liver by approximately 24 h, whereas the positive tumor retained signal for approximately 96 h. Cell labeling was observed as early as 2 min and gradual tumor cell internalization was observed over a 7 h period. These results suggest that antagonist behaving ligands, like Dmt-Tic-Cy5, that quickly bind with high affinity to the cell-surface but do not stimulate receptor signaling, will eventually be taken into the cells in a matter of hours by a non-receptor mediated mechanism. This study demonstrates the potential for the use of the Dmt-Tic

ligand as a targeted agent for in vivo imaging and the ability to characterize the in vivo properties of targeted fluorescent agents at the micro- and macro-scopic scales.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

BD	biodistribution
δOR	delta opioid receptor
DWC	dorsal window chamber
FOE	fold of enhancement
GFP	green fluorescent protein
h	hour
i.v	intravenous
NIR	near infrared
PK	pharmacokinetics
RFP	red fluorescent protein
ROI	region of interest
RT PCR	reverse transcriptase polymerase chain reaction

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Figure 1.

Characterization of the expression of the δ OR on human colon carcinoma cell lines. A) δ OR (*OPRD1*) mRNA expression in the parental HCT 116 and HCT 116/ δ OR cell lines, quantified by qRT-PCR and normalized to β -actin mRNA expression (* indicates p-value <0.05, n=3). B) One point binding of Eu-DPLCE ligand to δ OR in HCT 116/ δ OR cells compared to the parental cell line (* indicates p-value <0.0001, n=4). C) Total specific binding curve from Eu-DPLCE saturation binding assay (total minus nonspecific). Nonspecific binding was determined in the presence of 10 μ M naloxone ($R^2=0.999$, n=4). D) Standard curve for Eu DPLCE ligand plotting the relationship between fluorescence and ligand concentration per well determined that $1.6 \times 10^6 \pm 1.1 \times 10^5$ δ ORs are expressed on the cell surface of HCT 116/ δ OR cells (n=6). Note: AFU stands for Arbitrary Fluorescence Units.

Figure 2.

The in vitro live cell labeling of tumor cells (HCT 116/ δ OR) with 2.5 nM Dmt-Tic-Cy5 probe. A) Representative time-course of live cell fluorescence images acquired 30–1030 min post administration of 2.5 nM Dmt-Tic-Cy5. Scale bar = 10 microns. B) The ratio of the fluorescence in the cytoplasm (inside) to the fluorescence on the cell membrane (outside) was quantified for individual cells at each time point (11–25 cells per day). The average ratio inside/outside ratio from experiments done on three separate days is graphed. Error bars represent the standard deviation. The graphed results show the increasing accumulation of Dmt-Tic-Cy5 probe in the cytoplasm (inside) of δ OR-expressing tumor cells from 30 to 430 min.

Figure 3.

Pharmacokinetic studies of Dmt-Tic-Cy5 agent in a bilateral $\delta\text{OR-}/\delta\text{OR+}$ tumor xenograft mouse model. A) Representative fluorescence images acquired from 0 to 24 h post-injection of 4.5 nmol/kg Dmt-Tic-Cy5 showing specific uptake and binding of the targeted probe to the $\delta\text{OR+}$ tumor (blue arrows) with no significant uptake in the $\delta\text{OR-}$ tumor (white arrows). Uptake of the probe in the kidney (red arrow) and liver (yellow arrow) is also observed at 3 h. B) Graph depicts the mean fluorescence signal obtained from the $\delta\text{OR-}$ tumors and $\delta\text{OR+}$ tumors over a time course of 0–168 h ($n=5$ to 13). Maximum fluorescence signal in the $\delta\text{OR+}$ tumors is observed at 3 h ($p\text{-value} < 0.0001$, $n=13$). Note: Graph is not in linear scale. C) Linear regression line fits of fluorescence image data representing the initial uptake and clearance rates for positive and negative tumors. The slope of the line fits were used to determine the rates (Table 2).

Figure 4.

Biodistribution studies following administration of 4.5 nmol/kg Dmt-Tic-Cy5. A)

Representative ex vivo fluorescence images at select time points. B) Graph depicts the

quantified ex vivo fluorescence signal obtained in the δ OR+ and δ OR- tumors; with significant uptake of fluorescence in the δ OR+ tumor compared to the δ OR- tumor at every time point ($n=5$, p -value <0.02). C) Graph depicts the quantified ex vivo fluorescence signal obtained in the liver and kidneys ($n=5$).

Figure 5.

Intravital fluorescence imaging of agent (red) clearance from tumor circulation and extravasation into tumor tissue. A) Images were acquired at 1, 1.5, 2 and 5 minutes post administration of Dmt-Tic-Cy5. Mean intensity values from ROIs drawn within the vessel (yellow) and immediately outside the vessel (green) at each time point demonstrate B) the rapid initial clearance of the bolus from circulation within 2 min and C) steady extravasation and penetration into the tumor during the short time course. Scale bar = 50 μ m.

Figure 6.

A) Time-course of intravital confocal fluorescence images (25 $\times$ magnification) of a δ OR negative DWC xenograft tumor (top row) and a δ OR positive tumor (second row) pre- and post administration of 45 nmol/kg Dmt-Tic-Cy5. Agent related fluorescence is shown in red. B) Mean fluorescence intensity values for the entire image at each time point are quantified and graphed. Scale bar = 50 μ m.

Table 1

Mean fold of enhancement (FOE) over time for the 4.5 nmol/kg Dmt-Tic-Cy5 dosage as measured by in vivo fluorescence imaging.

Time (h)	Mean FOE (+ tumor/- tumor)	SD	Cohorts (n value)	p value
0.05	2.4	2.6	3	0.0226
0.15	2.7	2.1	3	0.0089
0.25	2.7	0.7	13	1.9360E ⁻¹⁰
0.50	3.2	0.8	8	3.5251E ⁻⁰⁸
0.75	4.5	2.1	13	1.4736E ⁻¹¹
1	6.5	3.4	10	1.8227E ⁻⁰⁶
3	9.7	4.3	13	1.6235E ⁻¹¹
6	17.5	7.1	7	2.1679E ⁻⁰⁵
12	13.5	2.0	7	4.2199E ⁻⁰⁹
18	26.7	9.3	7	1.0675E ⁻⁰⁵
24	20.6	5.8	12	9.9606E ⁻¹⁰
48	8.9	1.8	5	0.0002
72	10.8	2.8	7	1.2932E ⁻⁰⁶
96	5.6	2.5	7	0.0012
168	1.8	0.9	7	0.1761

Rates of uptake and clearance of the Dmt-Tic-Cy5 agent in the δ OR $^-$ tumor and δ OR $^+$ tumor as determined from the slope of the linear regression line-fit of in vivo fluorescence imaging data (n=5).

Table 2

Tumor Type	Rate Type	Time (h)	Slope $\times 10^7$ (Efficiency/h)	Standard Deviation $\times 10^7$	R Squared	p value
δ OR $^-$	Uptake	0 to 0.25	211	52.8	0.89	0.0573
δ OR $^+$	Uptake	0 to 0.75	264	70.0	0.78	0.0195
δ OR $^-$	Clearance	0.25 to 24	-14.6	3.09	0.88	0.0179
δ OR $^+$	Clearance	3 to 96	-1.45	0.38	0.70	0.0093