ABSTRACT: The melanocortin 1 receptor (MC1R) is overexpressed in most melanoma metastases, making it a promising target for imaging of melanomas. In this study, the expression of MC1R in a large fraction of patients with melanoma was confirmed using mRNA and tissue microarray. Here, we have characterized the in vivo tumor and tissue distribution and pharmacokinetics (PK) of uptake and clearance of a MC1R specific peptidomimetic ligand conjugated to a near-infrared fluorescent dye. We propose an interdisciplinary framework to bridge the different time and space scales of ligand–tumor–host interactions: intravital fluorescence microscopy to quantify probe internalization at the cellular level, a xenograft tumor model for whole body pharmacokinetics, and a computational pharmacokinetic model for integration and interpretation of experimental data. Administration of the probe into mice bearing tumors with high and low MC1R expression demonstrated normalized image intensities that correlated with expression levels ($p < 0.05$). The biodistribution study showed high kidney uptake as early as 30 min postinjection. The PK computational model predicted the presence of receptors in the kidneys with a lower affinity, but at higher numbers than in the tumors. As the mouse kidney is known to express the MC5R, this hypothesis was confirmed by both coinjection of a ligand with higher MC5R affinity compared to MC1R and by injection of lower probe concentrations (e.g., 1 nmol/kg), both leading to decreased kidney accumulation of the MC1R ligand. In addition, through this interdisciplinary approach we could predict the rates of ligand accumulation and clearance into and from organs and tumors, and the amount of injected ligand required to have maximum specific retention in tumors. These predictions have potential to aid in the translation of a targeted agent from lab to the clinic. In conclusion, the characterized MC1R-specific probe has excellent potential for in vivo detection of melanoma metastases. The process of cell-surface marker validation, targeted imaging probe development, and in vitro, in vivo, and in silico characterization described in this study can be generally applied to preclinical development of targeted agents.

KEYWORDS: malignant melanoma, Melanocortin 1 receptor, targeted imaging agent, pharmacokinetics, biodistribution
used intraoperatively for SLNB. The radiocolloid and dyes are not tumor-specific and, hence, cannot directly detect lymph node involvement.

Standard imaging techniques have been used for detection of distant metastasis, including CT, MRI, and 18F-FDG PET. However, all of these techniques have limitations [reviewed by authors in refs 11 and 15]. 18F-FDG PET lacks sufficient sensitivity to detect micrometastasis, especially for the assessment of SLNs. In addition, this tracer cannot discriminate between malignancy and inflammation, including nonneoplastic postoperative changes. Also, some normal tissues can accumulate 18F-FDG, including bowel, bladder, muscle, and brown fat, contributing to background signal. Specificity could be improved through the use of a molecular imaging probe targeting a cell-surface receptor that is highly and densely expressed in melanoma metastases relative to surrounding tissues.

Therefore, melanoma-specific molecular imaging probes are needed for the noninvasive and sensitive detection of regional lymph node involvement for staging of melanoma. In addition, they can be used to follow therapy response. Such probes could also be used for the intraoperative margin assessment and detection of regional lymph node involvement during surgery, and there is increasing interest in the use of novel targeted therapies for melanoma, which is notoriously resistant to most systemic therapies. For example, by attachment to a targeted agent with a cleavable linker, cytotoxins may be targeted to tumor cells for receptor-mediated endocytosis and intracellular release, increasing the effective local concentration at the metastasis, while maintaining tolerable systemic levels of cytotoxin, that is, increasing the therapeutic window.

Melanoma progression is associated with altered expression of cell surface proteins. It has been estimated that over 80% of melanomas express high levels of the Melanocortin 1 receptor (MC1R). MC1R is a member of a family of five G protein-coupled melanocortin receptors (MC1R–MC5R), which bind melanocyte-stimulating hormone (MSH) and related ligands. Because of the high expression of MC1R in melanoma, MSH has been investigated as a ligand for selective imaging and therapeutic agents, and a number of ligands have been developed. Off-target binding inside the melanocortin receptor family is undesirable given the presence mRNA coding for MC5R in human and mouse kidneys. Since the kidney is involved in agent clearance, MC1R selective ligands are needed. The other two MSH avid melanocortin receptor isoforms (MC3R and MC4R) are primarily expressed in the brain and CNS, which is protected by the blood–brain barrier.

In this study, we evaluated the expression of MC1R through RNA expression microarray and immunohistochemistry (IHC) analysis in melanoma patient samples and cell lines. While MC1R is not a novel target, this represents the most extensive study on its distribution in melanoma to date. In addition, a high affinity MC1R selective ligand with lower affinity for MC5R has been described, and we have recently modified this ligand with moieties to facilitate attachments. More recently, we have conjugated near-infrared fluorescent (NIRF) dyes to this ligand and demonstrated in vivo specificity for tumors with MC1R expression.

To characterize the biodistribution and the tumor–host–ligand interactions for these novel NIRF probes, we used an interdisciplinary approach: first, we used intravital confocal fluorescence microscopy of a dorsal skin-fold window-chamber mouse xenograft tumor model to demonstrate probe diffusion from blood vessels into tissue and uptake into tumor cells; then, in a higher time and spatial scale, in vivo time-resolved fluorescence imaging was used to characterize the heterogeneity of tumor uptake, biodistribution, and pharmacokinetics of the probe; and finally, a novel compartmental mathematical model was applied to interpret the experimental data and to estimate the pharmacokinetic parameters for the whole animal. These studies demonstrate that our probe has excellent potential for in vivo detection of melanoma metastasis and that the combination of quantitative imaging with PK mathematical modeling can be used to better characterize the behavior of targeted agents in vivo, simplify the interpretation of an otherwise complex system of multiorgan interactions, and shorten the gap between preclinical and clinical studies.

### EXPERIMENTAL SECTION

**Cell Culture.** A375 human malignant melanoma cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (Life Technologies), 100

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**Table 1. IHC Scoring of MC1R Expression in Patient Tissue Samples**

<table>
<thead>
<tr>
<th>tissue type</th>
<th>n</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>6</th>
<th>9</th>
<th>% ≥ 4</th>
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<tr>
<td>normal skin</td>
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<td>0</td>
<td>1</td>
<td>5</td>
<td>13</td>
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<tr>
<td>compound nevi</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>33</td>
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<td>0</td>
<td>4</td>
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<td>1</td>
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<td>20</td>
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<tr>
<td>intradermal nevi</td>
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<td>0</td>
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<td>22</td>
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<td>0</td>
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<td>7</td>
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<td>0</td>
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<td>6</td>
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<td>3</td>
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<td>1</td>
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<td>5</td>
<td>0</td>
<td>4</td>
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**Molecular Pharmaceutics**

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units/mL penicillin, and 100 μg/mL streptomycin in 5% CO2 at 37 °C. The cell line was obtained from American Type Culture Collection (ATCC), expanded for two passages, and cryopreserved. A375 cells engineered to express MC1R (A375/MC1R) were cultured in media containing 300 μg/mL of G418 for selection. All experiments were performed with cells of passage number less than 25. Cells were authenticated as negative for mycoplasma by testing at the ATCC and were monitored by microscopy and confirmed to maintain morphological traits over subsequent passages.

**DNA Microarray Analysis.** RNA extracts of melanoma cell lines were analyzed using the Affymetrix U133A array platform. Data generated from these arrays have been published previously and have been deposited in the NCBI Gene Expression Omnibus (GEO). Data are accessible using GEO Series accession GSE4845. For patients, affymetrix expression data for MC1R in patient tissue samples were compiled from publicly available data sets and the analysis was done as described before.

**Immunohistochemistry (IHC) of Melanoma Tissue Microarray (TMA).** A TMA was constructed at the Moffitt Tissue Core containing patient tissue samples of formalin-fixed and paraffin-embedded (FFPE) cutaneous specimens, including normal skin, a range of different types of nevi, primary melanomas and melanoma metastases (Table 1). The same method was previously reported for construction of a Ewing sarcoma TMA at the Moffitt Tissue Core Facility, except that the melanoma TMA has only one sample per case due to the large number of cases. No identifiable human subject information was associated with the melanoma TMA. Rabbit MC1R polyclonal antibody (GTX70735, GeneTex), 1:200 dilution, was used for staining with diaminobenzidine (DAB). The slides were scanned and scored as described before.

**Intravitral Imaging of the Dorsal Skin-Fold Window Chamber Tumor Xenograft Model.** A previously described dorsal skin-fold window chamber tumor xenograft model was used to study the pharmacokinetics of extravasation, tumor penetration, and tumor cell binding and uptake of the MC1R specific ligand conjugated to Cy5 NIRF dye (MC1RL-Cy5), except that A375/MC1R cells were used to form a tumor and the cells were mixed with GFP-expressing microvessel fragments from rat. Seven days after implantation of microvessels and tumor cells, mice were intravenously injected with 100 μL of 5% 10 000 MW cascade blue dextran (Invitrogen) in sterile H2O to verify microvascule patency. Then, 5 nmol/kg of the MC1RL-Cy5 probe was injected into the tail vein. Confocal images of probe uptake into the melanoma tumor cells were continuously acquired prior to, during, and after injection of the probe using the Olympus FV1000 (MPE) Multiphoton Laser Scanning Microscope (Lisa Muma Weitz Advanced Microscopy and Cell Imaging facility at USF) using 4× U-Plan Apo 0.16NA and 25X XL Plan N 1.05 NA water immersion lenses. The presence of MC1RL-Cy5 was measured by excitation with a 635 nm wavelength IR laser, and the emitted light was detected using a 655–755 nm filter.

Fluorescence image intensities were quantified using Image Pro Plus v6.2 (Media Cybernetics, Bethesda, MD). The histogram count/size selection tool was used to analyze groups of 3–4 cells in triplicate for 0 and 24 h time points following ligand injection. A threshold was set (R 10–255; G 0–255; B 0–255) to quantify the pixels positive for presence of the MC1RL-Cy5 probe. The Cy5 (R) channel intensity area and average intensity values were evaluated to determine the mean, standard deviation, and P-value by single tailed, paired Student’s t test.

**In Vivo Time-Resolved Fluorescence Imaging and Time-Domain Based Fluorescence Tomography.** Female nu/nu mice 6–8 weeks old (Harlan Sprague–Dawley, Inc., Minneapolis, IN) were injected subcutaneously with 1 × 10^6 MC1R expressing A375 cells in the right flank and parental melanomas in the left flank. Tumor volume was determined with calipers using the formula: volume = (length × width^2)/2. Once tumors reached 500–800 mm^3, 1–30 nmol/kg of the MC1R specific ligand conjugated to IRDye800CW from Li-Cor (MC1R-IR) in 100 μL of sterile saline was injected into the tail vein. In vivo fluorescence lifetime images were acquired using the Optix-MX3 (Advanced Research Technologies, Inc. a subsidiary of SoftScan Healthcare Group, Saint-Laurent, Canada). Animals were positioned on a heating pad and anesthetized using isoflurane (flow 2–2.5 L/min). Images were acquired using a scan resolution of 1.5 mm and a 790 nm pulsed laser diode with 40 MHz frequency and 12 ns time window. Images were analyzed using Optix-MX3 Optiview Software (version 3.01). The mean tumor fluorescence signal was determined prior to injection, then subtracted from mean normalized intensity values of postinjection ROIs, and tomographic image reconstructions were generated using the time-domain information from fluorescence lifetime scans.

**Ex Vivo Studies.** Tumors and kidneys were excised, and a ~1 mm center section was cut from each tumor while maintaining registration with the in vivo position of the tumor as best as possible. The center section was imaged ex vivo using both the Optix-MX3 and IVIS 200 (Caliper LifeSciences, Hopkinton, MA) imaging systems. For IVIS 200 acquisitions, the standard ICG excitation and emission filter set was used for imaging. After imaging, the crude center sections were fixed in formalin and embedded in paraffin for histology and fine sectioning. Formalin-fixed sections (5 μm) were IHC stained with MC1R primary antibody as described above for TMAs.

**Biodistribution Studies.** Mice were imaged and euthanized at 2–72 h postinjection (3 nmol/kg) time-points. Tumors, kidneys, and liver were excised, rinsed with PBS, blotted dry, and then imaged ex vivo with the Optix-MX3 as described above. Images were analyzed as described above.

**Mathematical Modeling.** In this work, we developed a new computational multicompartmental model to represent the accumulation and clearance of the ligand in the high MC1R-expressing tumor, kidney, bloodstream, and low MC1R-expressing tissue. This model was based on concepts introduced by Gabrielson and Weiner. We assumed conservation of the mass of ligand, which would only be excreted through the kidneys. The volumes of kidneys, high MC1R-expressing tumor, blood, and mouse obtained from ex vivo experiments were used in the computational model to determine the size of each compartment.

The computational model of the tumor is composed of two compartmens: the first represents the specific binding to the MC1R receptor, while the second represents nonspecific tumor uptake. The specific binding compartment of the tumor is described by three constants: $K_{nu}$, $K_{u}$ and $T_{int}$ which are the rates of probe accumulation, clearance, and the maximum concentration at saturation, respectively.

The non-MC1R specific tissue of the mouse (muscle, liver, etc.) was represented by one unique compartment, which accumulates and clears the ligand at rates $K_{ui}$ and $K_{u}$.
respectively. These two constants were also used to describe the nonspecific uptake compartments of the tumor and the kidneys.

The accumulation of ligand in the kidneys suggested that there might be some interaction between the ligand and kidney cells. To test this hypothesis, we included in the model a melanocortin-receptor specific binding compartment in the kidney, represented by the constants $K_{\text{Ki}}$, $K_{\text{Kor}}$ and $K_{\text{max}}$, meaning the rate of accumulation of ligand in the kidney, the rate of clearance of the ligand back to the bloodstream, and the maximum concentration of ligand at saturation, respectively. Both the specific and nonspecific compartments of the kidney were modeled to filter the ligand into the bladder at a rate, $K_{\text{Ko}}$.

The model was simulated by numerically solving a system of six difference equations (Supplementary eq 1) with time steps of 0.005 h (18 s). We implemented a Genetic Algorithm (G.A.) using the Java library JGAP (http://jgap.sourceforge.net/) to fit the model parameters to the experimental measurements $ex vivo$ and $in vivo$. Briefly, this algorithm creates a random group of possible fit solutions (500), and for a number of generations (1000) it evaluates the goodness of fit of the different solutions, attributing the highest scores to those with the best fit (shortest distance between the simulated curves and actual measurements). The best solutions increase their frequency in the pool of possible solutions, while the lowest scores are removed. Spontaneous "mutations" of these parameter values gradually change these solutions to add diversity to the population, and increase the chances of finding the best possible fit. The final product of this genetic algorithm is the set of pharmacokinetic parameters (Table 2) which, when incorporated into the mathematical model, generates ligand distribution curves which best fit the $ex vivo$ and $in vivo$ measurements.

**Statistics.** Data are represented as mean ± s.d. All statistical analyses were performed with GraphPad Prism version 5.01. An unpaired Student’s $t$ test was used to determine the statistical significance of differences between two independent groups of variables. For all tests, a $P \leq 0.05$ was considered significant.

### RESULTS

**MC1R Expression in Patient Tissue Samples.** It has been estimated that 80% of malignant melanomas express high levels of MC1R.\textsuperscript{24} For further confirmation and to characterize mRNA expression in patient tissue samples, we analyzed publicly available DNA microarray data sets, which showed that MC1R mRNA expression was highly and generally expressed in a large fraction of melanomas (Figure 1A, note log scale). In contrast, MC1R expression was not elevated in other skin cancers, normal skin, and organs involved in toxicity and drug clearance, that is, heart, lung, spleen, liver, and kidney.

To determine MC1R protein expression in patient samples, IHC was performed on a melanoma tissue microarray containing 267 samples. Figure 1C shows representative staining in normal skin relative to staining in a primary cutaneous melanoma, distant metastasis, and lymph node metastasis. None of the normal skin samples ($n = 19$) had staining with a pathology score of $\geq 4$, that is, homogeneous moderate to high staining (Table 1). Benign lesions ($n = 65$) had percentages of $\geq 4$ staining ranging from 15 to 33%. A relationship between primary cutaneous melanoma lesion size and pathology score was observed, with smaller lesions ranging from melanoma in situ to lesions 1 mm in thickness scoring 18% to 33% $\geq 4$, and lesions from 1 mm to $>4$ mm in thickness scoring 46–79% $\geq 4$. Primary mucosal melanomas ($n = 11$) and melanoma distant metastases had scores ranging from 40% to 67% $\geq 4$.

**MC1R Expression in Melanoma Cell Lines.** MC1R expression was examined on a previously published mRNA expression microarray of a panel of melanoma cell lines (Figure 1B).\textsuperscript{36,43} Moderate-to-high expression was observed in all primary melanocytes, and NRAS-mutant melanoma cell lines had low to moderate expression. In contrast, expression in $BRAF$ mutant melanoma cell lines was highly heterogeneous, with some lines exhibiting very low expression (WM 858, 793, 1799, 35) and some with extremely high expression (WM 164, 1727A, 1819, 239A). Notably, 3 of the 4 high expressing lines were metastatic: WM164, WM239A, and WM1727A.

**In Vivo Tumor Cell Uptake Studies.** We have recently demonstrated $in vivo$ tumor selectivity and $in vitro$ tumor cell uptake of NIRF dye labeled probes with high affinity and selectivity for MC1R.\textsuperscript{34,35} A375 melanoma cells transfected to stably overexpress MC1R with receptor number of 75 000 per cell were used as positive cells with high expression\textsuperscript{35} and parental A375 cells with endogenous expression were used as low-expressing cells.\textsuperscript{44} The high expressing A375/MC1R cells were used herein to form dorsal-window chamber (DWC) xenografts for intratral confocal fluorescence microscopy to demonstrate tumor cell uptake $in vivo$.

Since standard microscopy (metal halide) excitation light sources are inefficient at longer wavelengths and the commercially available fluorescence microscope detectors are generally inefficient at 800 nm wavelengths, we used the previously reported Cy5 dye conjugate (MC1RL-Cy5)\textsuperscript{35} for use in intratral fluorescence microscopy studies. The MC1RL-Cy5 probe has high affinity for MC1R, with a $K_i$ of 0.3 ± 0.05 nM.\textsuperscript{35} The binding affinity is comparable to that of unlabeled ligand, 0.24 nM $K_i$\textsuperscript{34}.

To study the distribution of the probe into tumors and tumor cells by intratral fluorescence microscopy, xenograft

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**Table 2. Parameter Value Estimates by the Multicompartmental Pharmacokinetic Computational Model Derived from $Ex Vivo$ Experiments**

<table>
<thead>
<tr>
<th>parameter\textsuperscript{a}</th>
<th>mean ± s.d. of 10 simulations</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_0$ (NU$^\text{−1}$ h$^{-1}$)</td>
<td>$2.7 \pm 1.4$</td>
</tr>
<tr>
<td>$K_0$ (h$^{-1}$)</td>
<td>$26 \pm 12$</td>
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<tr>
<td>$K_0$ (NU$h^{-1}$)</td>
<td>$1 \times 10^{-2} \pm 6.8 \times 10^{-3}$</td>
</tr>
<tr>
<td>$K_0$ (h$^{-1}$)</td>
<td>$7.8 \times 10^{-2} \pm 1.4 \times 10^{-2}$</td>
</tr>
<tr>
<td>$V_B$ (cm$^3$)</td>
<td>$1.4^b$</td>
</tr>
<tr>
<td>$V_T$ (cm$^3$)</td>
<td>$0.8 \text{ cm}^{16}$</td>
</tr>
<tr>
<td>$V_K$ (cm$^3$)</td>
<td>$0.9 \text{ cm}^{16}$</td>
</tr>
<tr>
<td>$V_N$ (cm$^3$)</td>
<td>$25 \text{ cm}^{16}$</td>
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<tr>
<td>$K_{\text{Ki}}$ (NU$^\text{−1}$)</td>
<td>$1.8 \pm 0.2$</td>
</tr>
<tr>
<td>$K_{\text{Kor}}$ (h$^{-1}$)</td>
<td>$2.3 \pm 0.6$</td>
</tr>
<tr>
<td>$K_{\text{max}}$ (NU)</td>
<td>$130 \pm 9$</td>
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<tr>
<td>$K_{\text{max}}$ (NU)</td>
<td>$2.7 \times 10^0 \pm 2.3 \times 10^3$</td>
</tr>
<tr>
<td>$K_{\text{max}}$ (h$^{-1}$)</td>
<td>$0.96 \pm 0.14$</td>
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</table>

\textsuperscript{a}Each parameter is defined in the Experimental Section. \textsuperscript{b}Denotes parameters whose values were obtained from weighing the mouse organs $ex vivo$ and were fixed in the beginning of the simulation; thus no standard deviation exists. $V_B$ is the volume of blood, $V_T$ is the volume of the MC1R high expressing tumor, $V_K$ is the combined volume of both kidneys, and $V_N$ is the total volume of the mouse minus kidneys, blood and MC1R+$^+$ tumor. NU stands for the Normalized Unit of fluorescence measured, which is proportional to ligand concentration.

---
tumors were implanted under the glass cover of dorsal skin-fold window chambers (Figure 2A). The tumors were constructed using A375/MC1R cells mixed with GFP rat microvessels in type I collagen. Following a 5–7 day period of tumor growth,
microvessel patency was verified by i.v. injection of blue dextran and regions of the tumor with patent GFP vessels were chosen for study (Figure 2B). extravasation, tumor cell binding, and uptake of the probe were observed by continuous confocal microscope acquisitions after i.v. injection of 5 nmol/kg MC1RL-Cy5 probe. Figure 2C shows the whole tumor surrounded by GFP microvessels at 24 h postinjection of probe (red) using low magnification (4×). Different postinjection time-points are shown in Figure 2D using higher magnification (25×). Immediately following injection, the probe was observed to extravasate and penetrate into the tumor. Probe fluorescence was observed at the periphery of cells as early as 5 min after injection. At 5 min to 2 h, probe fluorescence increased on the tumor cell surfaces, with some apparent cell uptake. At 24 h postinjection, the tumor cells had fully internalized the probe, unbound circulating probe had mostly cleared from circulation within the tumor, and significant fluorescence intensity was retained, p = 0.025, relative to preinjection images (Figure 2E).

Heterogeneity of Probe Uptake into Tumor. To investigate the heterogeneity of probe uptake in vivo, bilateral subcutaneous high and low MC1R expressing xenograft tumors (A375/MC1R engineered and A375 endogenous) were established. After tumor growth to approximately 500–800 mm³, the previously reported IRDye800CW conjugate (MC1RL-800) was injected intravenously, and fluorescence accumulation was monitored over time. The MC1RL-800 probe has high affinity for MC1R, with a 0.4 ± 0.1 nM Kᵢ which is comparable to that of unlabeled ligand, 0.24 nM Kᵢ. At 2 h postinjection, A375 tumors with low MC1R expression had significantly lower normalized fluorescence signal compared to A375/MC1R tumors (P ≤ 0.05, n = 3) (Figure 3A). In vivo tomographic slices were generated based on the fluorescence time-domain shift showing that probe distribution is heterogeneous in different slices of the tumor, where it has the highest accumulation depth from the top of the tumor (Figure 3B). Ex vivo images of the corresponding center sections of the high- and low-MC1R expressing tumors confirmed the in vivo results. IHC staining confirmed the high and low MC1R expression in the two tumor types, and areas with the highest IHC staining corresponded to areas with the highest fluorescence signal (Figure 3C).
**Figure 4.** Mathematical modeling of MC1RL-800 uptake in the body. (A) Graphical representation of the multicompartment model used in this study. The targeted probe injected into the bloodstream diffuses to all tissues in the mouse (NS Tissue), preferentially accumulating in the positive tumor in a specific and nonspecific manner (Tumor SB and Tumor NSB). Targeted probe also accumulates in the kidney in a specific and nonspecific way (Kidney SB and Kidney NSB), and is filtered into the bladder. (B) Multicompartmental model simulations of the pharmacokinetic distribution of probe over time at a range of dosages. On the left, the ex vivo imaging data for an injection of 3 nmol/kg were used to determine the model parameters with the best fit, the simulation (lines) overlay the data (points). In the middle and right are simulations of lower 1 nmol/kg and higher (10 nmol/kg) injected doses, respectively.

**Biodistribution Studies.** For biodistribution studies, mice bearing high expressing (A375/MC1R) tumors were injected with probe (n = 3), and tissue distribution of fluorescence signal was determined after removing tumors and organs from 30 min to 72 h postadministration (Figure 3D). At 30 min postinjection, probe was retained at relatively high levels in the MC1R high expressing tumor. At early time points, probe accumulation in the kidneys was significantly higher than in the tumors, for example, at 2 h after injection, kidney signal was 2 fold higher (P < 0.001). However, the difference in accumulation was no longer significant by 72 h after injection, and the probe was fully cleared from both tumors and kidneys by 96 h after injection. MC1RL-800 did not accumulate in the liver, and no signal was detected in the other organs, such as spleen, heart, brain, and so forth.

**Estimation of Whole Animal Pharmacokinetics Parameters.** A compartmental mathematical model was used to estimate the pharmacokinetics of probe uptake and clearance. The model includes tumor, kidney, and whole mouse compartment volumes and assumes mass conservation of the probe (Figure 4A). This model was used to account for the interference of tumor probe release in the uptake and clearance dynamics of blood and kidneys. The fit of ex vivo data for the 3 nmol/kg dosage in Figure 4B (left) shows a statistically significant correlation between experimental and simulated dynamics in two of the three measurable compartments: tumor (slope = 0.9298, R² = 0.9163), kidneys (slope = 0.8371, R² = 0.9370), and negative tissue (slope = 0.3371, R² = 0.7040), (Supplemental Figure S1). The poor correlation between computer simulations and actual measurements in the negative tissue was mainly due to the fluorescence levels measured being closer to background.

The parameters obtained from the model (Table 2 and Supplementary Figure S1) help clarify the dynamics of this system: the rate of accumulation of probe is faster in the high expressing tumor compared to the kidneys in the specific binding compartments (Table 2), and the tumor has a higher affinity for the ligand (Kₐ = 8.7 ± 0.5) than the kidneys (Kₐ = 40 ± 17) (Supplementary Figure S1), which explains why the model predicts that the probe reaches peak concentration in the tumor before reaching peak in the kidneys (30 min for the tumor, 2 h for the kidneys). Therefore, the PK computational model predicted the presence of receptors in the kidneys with a lower affinity (this prediction was confirmed by an experiment that will be explained below, Figure 5C).

The actual clearance of probe observed in the tumor and kidneys is the combination of the dynamics between negative tissue, tumor, kidneys, and blood. In the early moments after injection, most of the probe is present in the blood. At this point, the accumulation of probe in the tumor, kidneys, and negative tissue is determined by tissue-specific kinetic properties. However, once equilibrium is reached, the only outlet for this system is the rate of transport of the probe from the kidney into the bladder, Kᵦ, which should lead to a clearance with a half-life of ~9 h. The actual 20 h half-life observed in the kidneys is due to further uptake of probe from the MC1R expressing tumors and negative tissue, due to clearance through the blood, which in turn determines the clearance of these tissues (22 h for the tumor and 16 h for negative tissue).

**Effect of Probe Concentration on Uptake and Clearance.** To determine whether the amount of the MC1RL-800 probe injected had an effect on kidney uptake and retention, different amounts of the probe were injected i.v. and the pharmacokinetics of uptake and clearance monitored (Supplementary Figure S2). As expected, the lower dose (1 nmol/kg) provided greater discrimination and more rapid renal clearance compared to the higher dose (5 nmol/kg) (Figure 5A). Therefore, the renal uptake can be reduced by injection of
lower probe concentrations (e.g., 1 nmol/kg) which has higher affinity against MC1R, compared to MC5R (Ki values of the ligand against MC1R and MC5R are 0.24 nM and 46 nM, correspondingly). MC5R is expressed in kidney mouse (see below for details). Furthermore, with either dose, the measurable tumor and kidney half-lives were approximately 20 h for the kidneys and 22 h for the tumors. Following specific uptake of probe into cells, the fluorescent dye may be quenched in the acidic lysosomal environment. Hence, this half-life may represent a combination of agent clearance into the blood and acid quenching of dye following cellular internalization. This half-life is also dependent on the clearance of the ligand through the kidneys, into the bladder, which in the computational model was denominated Kd (Table 2). We plan to further characterize the rate of tumor clearance in future studies using Eu-labeled ligands, which cannot be acid-quenched.

The compartmental math model simulations also confirm the behavior observed in vivo when the probe is injected at increasingly higher concentrations: at low concentrations (Figure 4B, middle) the tumor and kidneys show similar probe levels, but at higher probe injection concentrations (Figure 4B, right), the kidney becomes increasingly brighter while the signal measured in the positive tumor and negative control tissue in the mice become closer (Figure 4B and Supplementary Figure S2).

**Effect of Differential Marker Isoform Affinities and Expression Levels on Probe Uptake and Clearance.** As it was mentioned earlier, the computational model predicted that the ligand has lower affinity for receptors in the kidneys compared to the receptors in tumors. As both human and mouse kidneys express MC5R29,30 and have little or no expression of MC1R, we hypothesized that the difference in kidneys and tumor is occurring via off-target binding of the probe to MC5R. IHC staining of a mouse kidney section shows strong expression of MC5R in the medulla (Figure 5B, right panel), and ex vivo fluorescence imaging of the corresponding kidney shows probe retention in the area of the medulla (Figure 5B, left panel). To test the hypothesis, 1 nmol/kg of an MC5R-selective ligand, H-Tyr-Val-Nle-Gly-His-DNal(2′)-Arg-DPhe-Asp-Arg-Phe-Gly-NH2, was injected. This agent has Ki values of 5.6 and 0.71 nM for MC1R and MC5R, respectively. Co-injection of the MC5R-specific compound along with 5 nmol/kg of MC1RL-800 significantly reduced renal uptake, that is, 36% decrease at 2 h after injection (p < 0.05), compared to control animals (Figure 5C), and the A375/MC1R tumors also had significantly lower signal following blocking (28% decrease at 2 h). Thus, it appears that the renal uptake of MC1RL-800 is due to off-target binding, which can be pharmacologically blocked.

In agreement, the computational pharmacokinetic model estimated the Kd (probe concentration needed for 50% saturation of target receptors) in the tumor (MC1R) and kidneys (MC SR) to be ~8.7 and ~40 units of normalized fluorescence, respectively, indicating that the tumor affinity for

**Figure 5. In vivo pharmacokinetics study of MC1RL-800.** (A) Plots of normalized fluorescence values for high- and low-expressing tumors and kidneys over a longitudinal time-course following administration of low (1 nmol/kg) and high (5 nmol/kg) doses of probe. Insets show representative normalized fluorescence intensity map overlays on visible light images of mice bearing bilateral flank xenograft tumors, 2 h post intravenous injection of the probe that were acquired using the Optix-MX-3 instrument. (B) A representative ex vivo fluorescence image (left) of kidney 2 h after probe injection acquired using the IVIS 200 instrument and corresponding IHC stained sections of MC1R (center) and MC5R (right). (C) Co-injection of 1 nmol/kg of MC5R specific ligand and 5 nmol/kg of MC1RL-800 (right image) to reduce kidney uptake of the probe. Inset shows representative normalized fluorescence intensity map overlays, 30 min post intravenous injection of the probe (control, left image) and probe plus MC5R ligand coinjection (right image). Images were acquired using the Optix-MX-3 instrument. Data represent mean ± s.d. NC: Normalized Counts.
studies have taken a patient tissue samples. It is not uncommon for protein the cell lines had comparable mRNA expression levels suggest that this may be an optimistic estimate, as only 67% of terminal lysine.

Several advantages, such as high biostability, easy synthesis and lysine insertion did not signifi cient alteration of ligand binding. This ligand to a NIRF dye with an excitation wavelength of 773 nm and emission wavelength of 792 nm (IRDye800CW Maleimide, LI-COR) to make 4-phenylbutyryl-His-DPhe-Arg-Lys(5-nynoyl)-NH\textsubscript{2} \textsuperscript{34} which was designed for attachment via a C-terminal lysine. \(K_i\) values for this ligand against MC1R and MC5R were 0.24 nM and 46 nM, respectively. The attachment and lysine insertion did not significantly alter ligand binding affinity or specificity. We have also described the attachment of this ligand to a NIRF dye with an excitation wavelength of 773 nm and emission wavelength of 792 nm (IRDye800CW Maleimide, LI-COR) to make 4-phenylbutyryl-His-DPhe-Arg-Trp-Gly-Lys(Mpr-IRDye800CW)-NH\textsubscript{2} (MC1RL-800) and confirmed the specificity of the probe by blocking experiment. Since longer wavelengths have lower tissue absorption and autofluorescence, this conjugate was used for in vivo fluorescence imaging studies. The conjugate retained high binding affinities comparable to the unmodified ligand. The use of small peptidomimetic ligands as carriers for the delivery of imaging or therapeutic moieties to diseased tissues offers several advantages, such as high biostability, easy synthesis and modification, faster blood clearance, high affinity and specificity, and low toxicity and immunogenicity. \textsuperscript{48}

In this study, tumor cell uptake was evaluated as an important factor for the optimal utility of targeted imaging and therapeutic agents using intravitral confocal fluorescence microscopy and showed the rapid extravasation and penetration of probe into the tumor, binding to the tumor cell surface at early time-points, and the complete cellular internalization and extracellular clearance from the tumor by 24 h after injection.

Endogenous MC1R expression ranges from several hundred to around 10,000 receptors per cell in different human cell lines.\textsuperscript{44} Our in vivo results indicate that the MC1RL-800 imaging probe is specifically retained in MC1R expressing tumors and normalized fluorescence counts distinguished A375 (400 ± 93 receptors/cell) from A375/MC1R tumors with engineered expression (75 000 receptors/cell). Although the difference in fluorescence values between high and low MC1R expressing tumors was not proportional to receptor numbers estimated by in vitro assays, it has been observed that protein expression in cell culture can be different when compared to expression in xenograft tumors made using the same cells.\textsuperscript{35}

Therefore, in vivo expression levels can be estimated by scaling. We have also reported the specific in vitro uptake of the MC1RL-Cys probe into B16F10 cells and in vivo retention of the MC1RL-800 probe in B16F10 tumors.\textsuperscript{45} B16F10 cells and tumors are melanotic and endogenously express MC1R at high levels (~22 000 ± 4200 MC1R/cell).

The in vivo biodistribution of MC1RL-800 probe indicates high tumor and kidney uptake as early as 15 min after injection. The kidney uptake was significantly decreased by coinjection of probe with an unlabeled ligand that has higher affinity for MCSR compared to MC1R or by injection of lower probe concentrations (e.g., 1 nmol/kg). These observations are in agreement with the in vitro binding data, confirming higher affinity of probe to MC1R compared to MCSR, and IHC data confirming MCSR expression in mouse kidney. When using nontoxic versions of the probe, for example, imaging agents, kidney accumulation should not be a serious problem, as complete clearance from the kidneys was observed within a few days (~96 h) after injection. However, the ligand may still have potential for tumor-specific delivery of cytotoxic agents through careful dose determination and use of MCSR specific blocking agents, or by regional administration in the form of isolated limb perfusion or infusion, which keeps the kidneys out of drug circulation and is used to treat in-transit melanoma metastasis.\textsuperscript{49}

The intratumor heterogeneity of the image reconstructions was confirmed by comparing the center slice from the in vivo images with the surface fluorescence measurement from the center section of the tumor ex vivo (Figure 3B). While not in perfect registration, the two images were similar in distribution of signal. Future studies will explore the possibility of using in vivo tomographic images for quantification of probe retention for longitudinal biodistribution studies, potentially eliminating the need to sacrifice multiple animals at a series of time points.

Our multicompartmental mathematical model correctly fit the empirical data of the dynamic uptake and clearance of probe from tumor, kidney, and melanocortin-receptor negative tissue, generating rate constants and maximum probe concentrations in the tumor and kidneys. The parameter values generated were independent of the administered probe concentration (dosage). By using the computational model, we were able to estimate the pharmacokinetic parameters of different tissues using measurements obtained from a complex in vivo system and use these parameters to understand the
interactions between ligand and tumor and ligand and host. This information will be invaluable for optimizing dosing and time points in future research with clinical trials.

The multicompartmental mathematical model presented in this work allows for a better understanding of the behavior of a targeted imaging probe in the mouse body, and quantitatively answers important questions such as: (1) how much probe should be injected in animal models and in future patients to maximize the tumor signal while minimizing nonspecific uptake in negative tissue, (2) what is the accumulation rate of a targeted probe in tumors, (3) what is the rate a targeted probe can clear from tumor and organs, and (4) what are the ideal time points for imaging. Therefore, the mathematical model developed in this study has the potential to aid in the translation of a targeted drug or imaging agent from lab to the clinic.

In conclusion, the imaging probe developed in this study has potential for the intraoperative detection of regional lymph node involvement and for margin detection in real-time during surgical removal of primary and metastatic melanoma lesions, potentially improving patient outcomes. In addition, the MC1R-specific ligand could be used to develop a PET or SPECT tracer for the noninvasive identification of regional lymph node metastasis and distant metastasis. The process reported herein that includes characterization of marker expression in patient samples, development of a marker-specific molecular imaging probe, and in vitro, in vivo, and in silico characterization of probe tumor specificity, pharmacokinetics and biodistribution is a framework that can be generally applied to preclinical development of targeted imaging and therapeutic agents.

**ASSOCIATED CONTENT**

Supporting Information

Scatter plot of experimental and simulations of model-predicted accumulation of ligand for positive tumor, negative tissue and kidneys, pharmacokinetic profiles of different probe concentrations, and multicompartmental whole body pharmacokinetic equations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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**REFERENCES**

20. Nguyen, Q. T.; Olson, E. S.; Aguilera, T. A.; Jiang, T.; Scadeng, M.; Ellies, L. G.; Tsien, R. Y. Surgery with molecular fluorescence imaging using activatable cell-penetrating peptides decreases residual


