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Molecular Cancer Imaging in the Second Near-Infrared Window Using a Renal-Excreted NIR-II Fluorophore-Peptide Probe

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Abstract

In vivo molecular imaging of tumors targeting a specific cancer cell marker is a promising strategy for cancer diagnosis and imaging guided surgery and therapy. While targeted imaging often relies on antibody-modified probes, peptides could afford targeting probes with small sizes, high penetrating ability and rapid excretion. Recently, *in vivo* fluorescence imaging in the second near-infrared window (NIR-II, 1000–1700 nm) showed promise in reaching sub-centimeter depth with micron-scale resolution. Here, we report a novel peptide (named CP) conjugated NIR-II fluorescent probe for molecular tumor imaging targeting a tumor stem cell biomarker CD133. The click chemistry derived peptide-dye (CP-IRT dye) probe afforded efficient *in vivo* tumor targeting in mice with a high tumor-to-normal tissue signal ratio (T/NT > 8). Importantly, the CP-IRT probes were rapidly renal excreted (~87% excretion within 6 h), in stark contrast to accumulation in the liver for typical antibody-dye probes. Further, with NIR-II emitting CP-IRT probes, urethra of mice could be imaged fluorescently for the first time non-invasively through intact tissue. The NIR-II fluorescent, CD133 targeting imaging probes are potentially useful for human use in the clinic for cancer diagnosis and therapy.

Tumor targeting probes specific to cancer biomarkers based on ligand-receptor mediated molecular recognition are of growing interest for *in vivo* imaging, early diagnosis of cancer

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and targeted therapy^[1]. As small molecular interface ligands, peptides provide cell penetrability, low immunogenicity, high biocompatibility and ease to synthesis. Nowadays peptides can be generated with prescribed binding characteristics to a broad range of targets including cancer biomarker proteins.^[2] Compared to antibodies, peptides can afford similarly high binding affinity and selectivity without complicated structures, and are advantageous in terms of smaller size and faster clearance from the body. Peptides can also be employed for chemical reactions in organic solvents not compatible with antibodies. Thus far, various peptides have been identified capable of specific binding to tumor biomarkers.^[3] Among them, affinity peptides and peptide analogs based therapeutics such as glucagon-like peptide-1 (GLP-1) are in clinical use or in active development.^[2a] Modern imaging technologies integrated with newly developed targeting imaging probes are widely used in *in vivo* tracking cellular events, diagnosing in the early stage, monitoring disease progression, imaging guided surgery and assessing therapeutic effect in none-invasive manners.^[4] RGD or NGR motifs have been successfully utilized as imaging based diagnostic agents,^[5] including radio-isotope modified targeting peptides for diagnosis by *in vivo* radio-tracing.^[6]

Peptide conjugated fluorescent probes have also been pursued for preclinical *in vivo* molecular imaging^[7]. Optical fluorescence imaging possesses the potential of high spatial and temporal resolutions, desired for basic biological research with animal models and subsequent clinical translation.^[7–8] However, traditional fluorescence imaging *in vivo* in the visible and NIR region of 400–900 nm has been superficial in depth with poor resolution, limited by tissue scattering of photons, autofluorescence and photo-bleaching.^[7] The poor imaging depth and low signal/background ratios have hindered fluorescence imaging in preclinical and clinical settings.

It has been shown that fluorescent probes emitting at longer wavelengths in the NIR-II window (1000–1700 nm) could benefit from reduced light scattering, allowing for deeper imaging depth into biological tissues.^[7, 9] Fluorescent imaging in NIR-II also benefited from diminished autofluorescence, affording improvements in spatial resolution at sub centimeter imaging depth with high signal-to-background ratios (SBR). Thus far, NIR-II fluorophores have included inorganic nanomaterials (i.e., carbon nanotubes, quantum dot and rare earth nanoparticles) and polymer or liposome encapsulated hydrophobic organic dyes.^[10] These nanoscale probes lacked the ability of quick excretion to alleviate safety concerns of exogenous foreign materials. Nanoparticle based NIR-II imaging agents are largely retained in the organs of the reticuloendothelial system (RES) such as the liver and spleen, and are excreted slowly through the fecal route. The long retention times have led to long-term safety concerns.

The majority of existing NIR-II probes exceeds the renal filtration threshold of ~ 40 kD or ~ 5 nm hydrodynamic diameter. We recently developed a small molecular dye CH-11055 with renal clearance ability.^[10d] However, the dye exhibited a low fluorescent quantum yield (~ 0.4% using IR-26 as a reference) in the NIR-II region. Upon conjugation to antibody or affibody, the complex size exceeded the renal cutoff and accumulated in the liver, causing a long retention of the dye.

Here, we developed a novel CD133 targeting peptide CP and conjugated it to the NIR-II emitting fluorophore IRT (Figure 1a), affording a CP-IRT NIR-II molecular imaging probe capable of renal excretion. The organic dye IRT contained donor-acceptor-donor core (D-A-D) and hydrophilic side groups,^[9c-e, 11] exhibited a QY of ~ 1.5% and could be renal excreted rapidly. It represented the brightest renal-excretion dye reported thus far with a fluorescence emission peak ~ 1050 nm (Figure 1) residing in the NIR-II region.

We chose the cell surface receptor CD133^[12] for molecular targeting (Figure 2). CD133 is a cholesterol-binding protein and an N-glycosylation-dependent epitope of the second extracellular loop of prominin-1.^[13] CD133 is overexpressed in many different types of carcinomas including glioblastoma, medulloblastoma, retinoblastoma as well as colon, liver, and ovarian cancer. As a common tumor marker, CD133 expression level is often investigated by *ex vivo* histological studies.^[14] Also, CD133 is a useful liquid biopsy biomarker for cancer diagnosis in methodologies such as circulating tumor cells (CTCs) detection.^[15] CD133 has also been found related to cancer stem cells (CSCs).^[16] Postnatally, CD133 protein is expressed by certain epithelial and mesenchyme cells, and by stem and progenitor cells of various organs. CD133+ cells found in these and other tumor types could self-renew, differentiate, and recreate the original tumors.^[17] Molecular imaging of the CD133 receptor could facilitate cancer detection for early diagnosis and assessment of cancer metastasis.^[14b] Recently, several CD133 ligands have been reported.^[18] However, *in vivo* NIR-II molecular imaging probes for CD133 with high biocompatibility and rapid excretion has not been demonstrated.

The CD133 targeting peptide CP (Figure 2a) was designed and synthesized with high affinity and high specificity by a screening technique^[10d] using a bi-model detection microarray described previously.^[19] CD133 have two large N-glycosylated extracellular loops, a leucine zipper motif and a transmembrane segment enriched in cysteine residue.^[20] Aromatic and basic amino acid residues could mediate electrostatic interactions with the sialic acid residues of the glycosylation epitope of CD133. In our design, we introduced mixes of amino acid residues placed at specific positions to create a 13-mer one-bead/one-compound (OBOC) library through a solid phase peptide synthesis (SPPS) strategy.^[21] The library contained CX₁X₂X₃X₄X₅X₆X₇X₈X₉X₁₀GM in which X represented either W, Y, F, R, K, H, Q, I, S, N, D, E, P, or L residues (see details in SI and Figure S1). The capacity of the peptide library was about 3×10⁶ with a redundancy of four for the library. Hence, a microarray high-throughput peptide screening was realized using approximately 1×10⁷ candidate beads. *In situ* MALDI-TOF-MS (Matrix-assisted laser desorption/ionization time of flight mass spectrometry) sequencing and fluorescence “turn-on” detection was performed (Figure S2a and b).^[22] By so doing, we screened out a series of positive sequences (Figure S2c) and mapped out the new CD133 targeting peptide named CP (see Figure 2a for chemical structure).

The binding affinity of CP towards CD133 were characterized by surface plasmon resonance (SPRi) imaging. The dissociation constant (K_D) was calculated to be ~ 7.37 nM from curve-fitting of real-time binding and washing data (Figure 2b). The low dissociation constant suggested high binding affinity of the CP peptide to CD133 protein receptors. To glean the interactions between CP and CD133, molecular docking simulations were performed (Figure

2c). The 3D coordinates of CD133 were obtained from the SWISS-MODEL.^[23] The molecular structure of CP was first optimized using *PEP-FOLD3*,^[24] and docking into CD133 was analyzed by *ZDOCK 3.0.2* (see binding energy calculation in SI and Table S1).^[25] The docking results showed that CP binding to the 644–777 site of CD133 through several mechanisms. The benzene rings of Trp2 on CP and Tyr753 on CD133 exhibited π - π interactions. The H atom in Arg5 (CP) and O in Ser760 (CD133) exhibited hydrogen bonding, similar to H in Arg5(CP) and O in Glu763 (CD133), and H in His7 (CP) and OH in Tyr749 (CD133). The amino acid N in Arg5 (CP) and O in Ser60 (CD133) exhibited electrostatic interactions. These effects synergistically led to highly specific CP-CD133 recognition with a small K_D .

To test the effectiveness of peptide CP targeting, we compared the specificity of cellular uptake of CP against a commercial anti CD133 antibody. The CP peptide labeled with fluorescein isothiocyanate (FITC) was used to target CD133+ colon cancer HT-29 cells, and found colocalized with P-phycoerythrin (PE) labeled CD133 antibody (Figure 2d upper). This suggested that the CP peptide binding site was a CD133 epitope. Moreover, we investigated competitive binding between CP peptide and antibody and found no obvious crossing effects, indicating different binding sites between the peptide and antibody (Figure S3). Further, we transfected HT-29 cells with CD133 short interfering RNA (siRNA) to knock down the expression of CD133. In this case, FITC-CP (1 mM) and PE-antibody (10 μ M) both showed diminished signals on the HT-29 cells due to the suppressed expression of CD133 receptor by RNA interference (Figure 2d bottom). Real-time polymerase chain reaction (RT-PCR) and western blot assay were carried out to confirm the successfully knock-down of the CD133 expression in HT-29 cells at the gene and protein levels, respectively (Figure S4). These results confirmed the binding affinity and specificity of CP peptide towards CD133 receptors. Furthermore, internalization study indicated that CP could first recognize the cell membrane and then penetrate into the cytoplasm effectively (Figure S5).

We further investigated the binding efficacy of CP against cancer stem cells (CSCs). CD133 proteins are expressed by certain epithelial and mesenchyme cells postnatally, by stem and progenitor cells from various organs, and by CSCs of many different types of malignant tumors. Recognition of CD133 epitope by CP probe could be a potential strategy for CSCs recognition-based diagnosis. Tumor stem cell sphere with differentiative potential was isolated from the cancer cell lines. We identified several stem cell markers on the tumor spheres^[26] that were significantly overexpressed (Figure S6). FITC labeled CP binding on the surface of the stem cell sphere highly co-localized with PE labelled CD133 antibody (Figure 2e). This indicated that we isolated the CD133+ CSCs with high-purity. We have calculated the co-localization coefficient of CP and antibody from the confocal images using *Image J* software. For the cell line, the Pearson's coefficient is calculated to be 0.25 and the overlap rate is 94%. For the stem cell sphere, the Pearson's coefficient is 0.34 and the overlap rate is 90%. It is indicated that both showed relatively high overlap rate (Figure S7).

We employed a renal excretable NIR-II molecular fluorophore IRT based on our recent work on donor-acceptor-donor intermolecular charge transfer complexes (Figure 1a schematic).^[27] The IRT dye contained polyethylene glycol (PEG) and tert (ethylene glycol) (TEG)

groups to impart high water solubility, and azide groups for functionalization using click chemistry.^[9c-e] We conjugated the IRT dye to CP peptide by click chemistry via a propargylglycine group attached to the N-terminal of CP peptide using TBTA (Tris (benzyltriazolylmethyl)amine)-copper (II) catalyst (Figure 1a, see SI for experimental details). The CP peptide-IRT dye conjugate exhibited an absorption peak ~ 750 nm, fluorescence peak of ~ 1050 nm (Figure 1b) and a quantum yield (QY) of ~ 1.5% based on the IR26 reference (QY~ 0.5% in organic solvent).

The CD133 molecular specificity of CP-IRT was first tested on a plasmonic gold (pGOLD) chip containing a micro-array of CD133+ HT-29 cell lysate and U87MG (human primary glioblastoma cell line) cell lysate, and CD133 negative HEK293T cell lysate micro-printed on pGOLD for reverse phase protein array assay.^[11a, 28] CP-IRT was incubated with the printed slide followed by washing and exposure to CP-IRT probes (see details in SI). We observed bright NIR-II fluorescence from both HT29 and U87MG spots and much weaker fluorescence from HEK293T spots, indicating high affinity of the CP conjugated NIR-II molecular probes for specific CP-CD133 recognition (Figure 3a). This result was further confirmed by molecular imaging of live cells *in vitro* (Figure 3b), with the cells nuclei labeled by a red nuclear stain DRAQ5 (Ex:647 nm; Em: 697 nm). Imaging was done using a home built confocal microscope covering a broad 650–1700 nm emission range (see SI for experimental details).

For *in vivo* molecular imaging, we investigated a mouse HT-29 tumor model using CP peptide-IRT fluorophore as the targeting probe. C57BL/6 mice inoculated with subcutaneous xenograft HT-29 human colon tumor (CD133+) were tail-vein injected with CP-IRT probes in PBS (50 μ M, ~ 200 μ L). All of our *in vivo* NIR-II imaging was performed by collecting fluorescence above 1200 nm under an 808-nm laser excitation with a typical illumination power of ~ 75 mW/cm². We observed clear vasculature structures under the skin of mice during the initial phase of CP-IRP circulation in the blood (Figure 4a). Homing and accumulation of CP-IRP to the CD133+ HT-29 tumor over time were observed over time, with the tumor to normal tissue (T/NT) signal ratio reaching a maximum of T/NT ~ 8.3 at 2 h post injection (p.i.) (Figure 4b-d). Parallel data of biodistribution showed consistency (Figure S8). In contrast, without the CP-peptide, the free, non-targeted IRT dye showed weaker signal in the HT-29 tumor and reached a maximum T/NT ~3 at 4 h p.i. (Figure S9-10), suggesting passive accumulation of the free dye in the tumor. Specific *in vivo* HT-29 tumor targeting by the CP-IRT probe was also verified by injecting a blocking dose of CP peptides 0.5 h before the CP-IRT injection. In this case low tumor fluorescence signal was observed 2 h p.i., suggesting blocking/saturation of CD133 receptors in the HT-29 tumor by free CP peptides (Figure 4e).

We monitored the excretion behavior of our CD133+ tumor targeting CP-IRT probes over time. Shortly after tail vein injection, NIR-II imaging revealed CP-IRT circulating to the urinary system. The bladder NIR-II fluorescence signal could be discriminated clearly from other tissues, and within ~ 20 min p.i. most of the targeting CP-IRT probes were excreted through the bladder (Figure 5a). To quantify excretion, urine was collected for 6 hours p.i. (Figure S11). The NIR-II fluorescence intensity (1200 nm long pass filter under 808 nm excitation) of each urine sample was analyzed to calculate the total amount of the excreted

dye using a standard calibration curve (see SI and Figure S12). Over 87% of the injected peptide-NIR II dye probes were excreted through the urine within 6 h post injection (Table S2-S4), suggesting an exciting NIR-II fluorescent molecular CD133 imaging probe that was highly effective in tumor targeting and could be rapidly eliminated from the body.

Also interesting was that within minutes post injection of CP-IRT, the two urethras of mouse could be clearly imaged non-invasively through the intact mouse tissue/body (Figure 5b). This was the first-time mouse urethra could be imaged by a fluorescence technique *in vivo* without surgical removal of tissues, owing to reduced tissue scattering of light in the long-wavelength NIR-II regime. The frozen section image was taken to further confirm the urethral uptake of CP-IRT (Figure S13). Hence, renal excretable NIR-II probes could open up a fluorescence-based imaging of mouse urethra useful for investigating animal ureter disease models.

We compared the excretion behavior of CP-IRT to that of protein and antibody conjugated IRT. Antibodies were large functional proteins relative to peptides with molecular weight ~ 100 kDa. We found that IRT dye conjugation with antibody and smaller proteins like bovine serum albumin (BSA) led to high liver uptake without renal excretion (Figure 5c). Dynamic light scattering measurements (Figure 5d) showed CP-IRT size of ~ 5 nm (within the renal excretion range), whereas BSA-IRT size was ~ 25 nm, well above the kidney filtration cutoff. Thus, peptide conjugate with a small organic NIR-II dye showed the advantage of rapid elimination from the major organs of the body, without the problem of long retention in the RES system often seen with antibody-dye probes or nanoparticle-based fluorophores.

Furthermore, pharmacokinetics and blood hemogram study were carried out after the CP-IRT injection. Pharmacokinetics study was performed by monitoring the circulating NIR-II signal in the superior sagittal sinus for several hours after CP-IRT injection.^[29] As shown in Figure S14, the blood circulation half-time of CP-IRT was $\sim 2.69 \pm 0.28$ hour. We also carried out routine blood examination and blood chemistry studies (Table S5-6). The hemogram values did not show marked difference compared to normal control, and blood chemistry results indicated that there was no liver or kidney damage after CP-IRT treatment.

In summary, we developed a new peptide CP targeting a cancer stem cell marker CD133 with high affinity ($K_d \sim 7$ nM) and specificity. A novel peptide conjugate with a NIR-II organic dye was produced for *in vitro* and *in vivo* molecular fluorescence imaging with a ~ 300 nm Stoke's shift. The click chemistry derived peptide-dye (CP-IRT) probe afforded efficient *in vivo* tumor targeting in mice with a high tumor-to-normal tissue signal ratio ($T/NT > 8$). Importantly, the CP-IRT probes were rapidly renal excreted (~ 87% excretion within 6 h), in stark contrast to accumulation in the liver for typical antibody-dye probes. With CP-IRT probes, the urethra could be imaged fluorescently for the first time non-invasively through intact mouse tissue. The high-safety, NIR-II fluorescent, CD133 targeting probes are potentially useful for human use in the clinic for cancer diagnosis and therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

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Figure 1. The structures and spectrum of CD133 targeting NIR-II dye-peptide probe CP-IRT. (a) The schematic of the water soluble IRT dye with donor-acceptor-donor core (D-A-D) structures and azide groups for functionalization using click chemistry as well as the conjugation route between IRT and propargylglycine-CP by TBTA-Cu (II) catalyzed method under the reduction by sodium ascorbate. (b) The UV-Vis-NIR absorption and photoluminescence (PL) emission (808-nm excitation) spectra of CP-IRT.

Figure 2. Peptide CP and CP affinity characterization at both molecular and cellular level in Vis-NIR region

(a) The chemical structures of CP. (b) SPRi detection of CP towards CD133. (c) The molecular docking of CP ligand and CD133 receptor: 1) the benzene rings of Trp2 (CP) and Tyr753 (CD133) have π - π interaction. 2) H in Arg5 (CP) and O in Ser760 (CD133) have hydrogen bond. 3) H in Arg5 (CP) and O in Glu763 (CD133) have hydrogen bond. 4) N in Arg5 (CP) and O in Ser60 (CD133) have electrostatic interaction. 5) H in His7 (CP) and OH in Tyr749 (CD133) have hydrogen-bond. 6) the aromatic rings of His7 (CP) and Tyr753 (CD133) have π - π interaction. (d) Co-localization of the FITC labelled CP (green channel) and PE labelled CD133 antibody (red channel) and the nuclei indicator Hoechst33342 (blue channel) of HT-29 cells, the upper row is HT-29 cells transfected with uncorrelated siRNA (not knockdown CD133) and the bottom row is HT-29 cells transfected with CD133 siRNA. (e) Co-localization of CP-FITC (green channel) and PE-CD133 antibody (red channel) and Hoechst33342 (blue channel) on a tumor stem cell sphere with differentiative potential.

Figure 3. The affinity and specificity testing of CP-IRT.

(a) NIR-II assay testing for CP-IRT conjugation. HT-29, U87MG, HEK293T cell lysates were printed on plasmonic fluorescence-enhancing gold slides for checking the CP-IRT. The slide was imaged with a 10× magnification NIR-II setup with 808-nm excitation and a 1100-nm long-pass emission filter. (b) The binding behavior of CP-IRT towards HT-29 (CD133 positive), U87MG (CD133 positive), HEK293 (CD133 negative) cells and the free dyes towards HT-29 cells. Imaged by a home-built Confocal setup with 785-nm excitation and a 1100-nm long-pass emission filter for membrane (red channel) as well as 658-nm excitation and an 850-nm long-pass emission filter for nucleus (blue channel).

Figure 4. In vivo fluorescence imaging of tumor-bearing mice intravenously injected with CP-IRT in NIR-II window.

(a) NIR-II fluorescence time points taken 0h-6h as well as 48h after an intravenous injection of CP-IRT with an 808-nm laser excitation through collection of fluorescence emitting using the 1200 nm long pass filter on a CD133+ tumor bearing mouse. (b) The digital photograph of the tumor bearing C57BL/6 mouse of which the blue border is the imaging view of filed (c) Profile of the fluorescent intensity across the mouse body including liver and tumor after CP-IRT injection. (d) Variation of T/NT signal ratio as a function of time post injection with CP-IRT. (e) NIR-II imaging of the CP-IRT injection after blocking by CP peptide only.

Figure 5. *In vivo* Molecular imaging with CP-IRT to monitor the excretion ability.

(a) Selected time points from video-rate NIR-II imaging (1200nm LP filter, 200 ms) of a mouse in the supine position after an intravenous injection of CP-IRT (b) High resolution urethra imaging by CP-IRT. (c) Selected time points from video-rate NIR-II imaging (1200nm LP filter, 200 ms) of a mouse in the supine position after an intravenous injection of Protein conjugated IRT injection showing disparate liver and bladder fluorescent signals (C57BL/6 mice). (d) Dynamic Light Scattering Measurement of IRT, CP-IRT and Protein IRT.