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Design of a ligand for cancer imaging with long blood circulation and an enhanced accumulation ability in tumors†‡

 Elnaz Nakhaei,^a Chan Woo Kim,^a Daiki Funamoto,^a Hikari Sato,^a Yuta Nakamura,^a
 Akihiro Kishimura, ^{abc} Takeshi Mori ^{ab} and Yoshiki Katayama^{*abcde}

The use of imaging agents to visualize tumor cells is an advantageous technique to achieve a more efficient intraoperative diagnosis and effective debulking operations. Targeting of these agents to certain receptors that are overexpressed in cancer cells, such as the folate receptor, aids in tumor targeting. Several imaging probes have been developed using this strategy. However, these ligand-targeting cancer imaging probes are rapidly cleared during systemic delivery due to their small size, which compromises their bio-distribution and circulation. Improving the detection of cancer requires higher accumulation and effective retention activities of imaging probes. Here we developed a new design for a folate-fluorophore conjugate that is modified with palmitoyl. Palmitic acid has a strong binding affinity with human serum albumin (HSA), which has the ability to form non-covalent host-guest complexes and has a blood half-life of 19 days. In this strategy, HSA is expected to serve as an endogenous nanocarrier for the designed probe in blood circulation. We hypothesized that *via* a reversible interaction with HSA, this simple palmitoyl modification on a folate-fluorophore conjugate can induce long blood circulation of the probe. Our folate-targeted probe could show longer blood circulation compared to the probe which lacks palmitoyl.

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1. Introduction

Tumor imaging has become an important technology in cancer diagnostics and prognosis. Many types of imaging molecular probes for various modalities, such as positron emission tomography (PET), magnetic resonance imaging (MRI), and computed tomography (CT), have been successfully introduced into clinical practice.^{1,2} Intra-operative diagnosis is another promising technique to visualize tumor regions in tissues. This technique should improve the efficiency of removal of tumor tissues during surgery, as it assists in real-time feedback in monitoring residual malignant tissues, thus contributing to improved overall survival.^{3–5}

In the development of intra-operative imaging techniques, fluorescence detection is one of the most promising modalities due to its high sensitivity and resolution. However, a fluorescence probe for intra-operative use has not been fully developed. The molecular probes should have some essential characteristics, such as high specificity against target cancer cells and sufficient accumulation and retention in the tumor tissue.

To ensure cancer specificity, tumor cell-specific targeting ligands are usually used.^{5–7} Thus, certain receptors, such as the glutamine transporter, EGFR or folate receptor, that are overexpressed in various cancer cells have emerged as optimal targets for imaging.^{8–10} However, imaging probes using targeting ligands are in general rapidly excreted from kidneys during systemic application due to their small size.^{3,8,11,12} This results in a demand for a higher dose of the probe and limited intraoperative surgery, as the surgery requires several hours. Thus, the size of the probe may be one of the critical factors for long circulation in the blood. Probes smaller than 5 nm are easily excreted through renal clearance, while probes larger than 500 nm are captured by the liver and spleen.¹² Therefore, fluorescence imaging probes should range in size between 5 and 500 nm. The size of a probe is also a key point for its specific accumulation and retention in the tumor. In normal tissues, well-organized blood vessels have a regular and integrated structure. However, in tumor

^a Graduate School of Systems Life Sciences, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka, 819-0395, Japan. E-mail: ykatatcm@mail.cstm.kyushu-u.ac.jp; Tel: +81 092 802 2850

^b Department of Applied Chemistry, Faculty of Engineering, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan

^c International Research Center for Molecular Systems, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan

^d Center for Advanced Medical Innovation, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan

^e Department of Biomedical Engineering, Chung Yuan Christian University, 200 Chung Pei Rd., Chung Li, Taiwan, 32023 ROC

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tissues, the neovasculature enhances extravasation of macromolecules that are smaller than 100 nm, and an incomplete lymph duct also contributes to the retention of such macromolecules in tumor tissues. This effect is known as the enhanced permeability and retention effect and has been used in many types of tumor delivery methods as a gold standard.^{13,14} However, from a practical viewpoint, such probes may have some drawbacks, such as difficulty with production and regulation of their characteristics.

This study focused on the improvement of the circulation ability and accumulation of a targeted fluorescent molecular probe for tumor imaging by using a small molecule. Human serum albumin (HSA; 66.5 kDa) is the most abundant plasma protein in the blood (*ca.* 0.6 mM, $\sim 42 \text{ g L}^{-1}$) and tissue fluids, with an average half-life of 19 days in blood circulation; HSA can also strongly bind with various small molecules.^{15,16} This binding property of HSA has resulted in research efforts to design and develop drugs with high binding affinity against HSA to either improve the pharmacokinetic profile or bioavailability of drugs.^{17–19} HSA also shows a relatively high uptake in tumor and inflamed tissues.^{20,21} Under cellular stress-inducing conditions, such as fast-growing tumors, cells preferentially take up HSA as the main energy source for growth and maintenance.²²

Considering these features of HSA, we hypothesized that a long blood circulation time and effective accumulation and retention in cancer cells would be rendered to a small fluorescence probe molecule that could bind with HSA.^{23,24} A long alkyl chain, which is a good ligand for HSA, was incorporated into a tumor-specific fluorescent small molecule. The probe also contains a folate (folic acid, FA) derivative to secure tumor specificity.

2. Experimental

2.1. Materials & reagents

NovaSyn TGR resin (amine density of 0.24 mmol g^{-1}) and all Fmoc-protected amino acids were obtained from Novabiochem, Merck (Tokyo, Japan). We obtained 1-hydroxy-1*H*-benzotriazole hydrate (HOBt·H₂O), *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU), diisopropylethylamine (DIPEA), dichloromethane (DCM), 1-methyl-2-pyrrolidone (NMP), *N*- α -(9-fluorenylmethoxycarbonyl)-*L*-glutamic acid α -*t*-butyl ester (Fmoc-Glu-*Ot*Bu), *N*- α -(9-fluorenylmethoxycarbonyl)-*N*- ϵ -palmitoyl-*L*-lysine (Fmoc-Lys(Pal)-OH), and piperidine from Watanabe Chemical (Hiroshima, Japan). *N,N*-Dimethylformamide (DMF) was purchased from Kanto Chemical (Tokyo, Japan). Dimethyl sulfoxide (DMSO), ethanol and E-MEM and D-MEM media were purchased from Wako Pure Chemicals (Osaka, Japan). RPMI 1640 (folate-free) medium was purchased from Life Technologies. We obtained 11-bromoundecanoic acid, 5-chloro-1-pentyne and 1,8-diazabicyclo[5.4.0]-7-undecene (DBU) from Tokyo Kasei Industry (Tokyo, Japan), and 5-(and 6)-carboxytetramethylrhodamine succinimidyl ester (NHS-Rhodamine) from Thermo Scientific. Sulfo-cyanine7 NHS ester was

obtained from Lumiprobe and Fmoc-8-amino-3,6-dioxaoctanoic acid (Fmoc-miniPEG) was obtained from Peptides International. *N*¹⁰-(Trifluoroacetyl) pteric acid, human serum albumin and folate (folic acid) were purchased from Sigma-Aldrich. All reagents were used without further purification.

2.2. Synthesis of the folate conjugates

Probes were synthesized by standard Fmoc-chemistry using the NovaSyn TGR resin (amine density of 0.24 mmol g^{-1}), DIPEA as a base, HOBt/HBTU as coupling reagents, and a 20% solution of piperidine in DMF for deprotection of the Fmoc group. First, Fmoc-Lys(palmitoyl)-OH was coupled onto the resin. Next, two miniPEG and a Lys(Boc) were modified as spacer and fluorophore modification sites, respectively. The folate moiety was modified with two-step introduction of glutamic acid and *N*¹⁰-(trifluoroacetyl) pteric acid. Since trifluoroacetyl, the protecting group of pteric acid, is resistant to general deprotection with trifluoroacetic acid (TFA), one additional deprotection step was conducted; the resin was washed with DMF five times and with DCM five times and then it was incubated with 1 mol L^{-1} ammonium hydroxide solution/DMF (1:1, vol/vol) for 30 min four times to cleave the trifluoroacetyl protecting group. The resin was washed three times with DCM and three times with *n*-hexane, and then dried under vacuum. Afterward, the conventional deprotection and cleavage from the resin solution were conducted by using TFA. To introduce the fluorescent group residue onto the lysine of the folate conjugate, Cy7-NHS ester in anhydrous DMSO (10 mg mL^{-1}) and DIEA were added to a peptide solution of 11.6 mg mL^{-1} in DMSO at a 1.2:1.2:1 dye, DIEA to peptide molar ratio. Then, the solution was mildly shaken for an overnight.

For the *in vitro* study, carboxytetramethyl rhodamine-NHS ester was used instead of Cy-7-NHS. Purification of the solid-phase synthesized peptides was carried out on an analytical reversed-phase high performance liquid chromatography (HPLC) system using a C18 RP ($250 \times 4.6 \text{ mm } 5 \mu\text{m}$) column using a linear A–B gradient at a flow rate of 1.16 mL min^{-1} , where eluent A was 0.1% TFA in water and eluent B was 0.1% TFA in acetonitrile. UV absorbance at 220 nm, 552 nm, and 740 nm was monitored.

2.3. Cell lines and cell culture

The epidermal mouth carcinoma HeLa-contaminated KB cell line that overexpresses the folate receptor (FR- α (+)) was cultured in E-MEM. The A549 cell line, which is negative for the folate receptor (FR- α (-)), was cultured in D-MEM. The media contained 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin, $100 \mu\text{g mL}^{-1}$ streptomycin, and 0.25 g mL^{-1} amphotericin B (all from Gibco Invitrogen Co., Grand Island, NY, USA). Cells were cultured in a humidified atmosphere containing 5% CO₂ and 95% air at 37 °C.

2.4. Probe binding assays

KB and A549 cells were seeded at a density of 5000 cells per well in a 96 well glass plate in 100 μ L of the RPMI 1640 (folate-free) medium. After 24 h, the wells were washed twice with 100 μ L of DPBS. Next, 100 nM of probe 1 in 100 μ L of the medium was added to the wells. The plate was incubated on ice for 30 min. The solution was removed and the cells were washed twice with 100 μ L of DPBS. Next, 100 μ L of the medium was added and the nuclei were stained with Hoechst 33342. After washing, the cells were observed by fluorescence microscopy. Images were obtained by using manufacturer-specified laser excitation wavelengths and emission filter sets.

2.5. *In vivo* mouse model

Animal studies were performed in accordance with the Guidelines for Animal Care and Use Committee at Kyushu University (Fukuoka, Japan). Female 5 week-old BALB/c nude mice were inoculated with a dorsal, subcutaneous injection of 1.6×10^7 KB cells and 6.5×10^7 A549 cells in 50 μ L of Matri (BD Biosciences, Bedford, MA, USA) and 50 μ L of PBS per animal. Tumors were allowed to grow for about two weeks to a mean diameter of approximately 10 mm.

2.6. Fluorescence imaging of tumor inoculated mice

Fluorescence imaging was performed using a cooled IVIS® CCD camera (Xenogen, Alameda, CA, USA) and analyzed with Living Image software. All fluorescence images were acquired with a 2 s exposure. For quantitative comparison, regions of interests (ROIs) were drawn over the tumor or the whole body and the results are presented as the mean $\pm$ standard deviation (SD) for a group of three animals. The excitation and emission maximum wavelengths of Cy7 are 740 nm and 773 nm, respectively. The most suitable filter in the IVIS® Imaging System covered the excitation passband of 710–760 nm and emission passband of 810–875 nm.

3. Results and discussion

3.1. Design and synthesis of the fluorescence probe interacting with HSA for cancer imaging

The structures of the synthesized probes are shown in Fig. 1. We hypothesized that introduction of a natural ligand for HSA to a fluorescence probe molecule would result in an enhancement of its blood circulation ability from its reversible interaction with HSA. This may be advantageous for *in vivo* stability compared with covalent bonding of the probe to HSA due to the lack of any denaturation or introduction of a non-natural structure, which may be recognized by the immune system. The C-terminus of the probe was modified by a palmitoyl group. Palmitic acid is naturally carried by HSA in blood circulation with a strong affinity.²⁵ For tumor targeting, we used the folate moiety, as various cancers show overexpression of the folate receptor. Between the folate moiety and the palmitoyl group, hydrophilic oligo-ethylene glycol was also incorporated to increase solubility and help the

Fig. 1 Chemical structure of the synthesized fluorescent probes 1–3. The R₁ and R₂ substituents in each probe are described in the inset table. Probe 1 was used in *in vitro* experiments and probes 2 and 3 were used in *in vivo* experiments. To introduce the palmitoyl group (pal) on the probe, the probe was modified on the side chain of a lysine.

binding of the folate moiety with its receptor on cancer cells. The molecule was successfully synthesized by solid phase synthesis with Fmoc chemistry.

For *in vivo* assays, the amine-reactive succinimide ester of Cy7 was incorporated in the probe. Cy7 has a high quantum yield of 20% and high photostability and is especially useful for near infrared (NIR) imaging. NIR Cy7 fluorescence imaging takes advantage of the transparency of biological tissues with a maximum excitation wavelength of 740 nm and a maximum emission wavelength of 773 nm. The obtained probe (Fig. 1, probe 2) and control probe (probe 3) were identified by MALDI-TOF-MS (Fig. S1†). We synthesized the Rhodamine-conjugated probe (probe 1) for the *in vitro* experiments in the same manner.

3.2. Demonstration of specific binding of the folate-NIR probe with confocal laser scanning microscopy

We first performed *in vitro* experiments to confirm the ability of the probe to specifically bind FR-expressing cells. FR (+) KB cells and FR (–) A549 cells were incubated with probe 1. As shown in Fig. 2, red fluorescence was only observed on the surface of FR (+) KB cells, confirming the successful binding of the probe to FR. No fluorescence was observed on FR (–) A549 cells, which confirms specific binding of the probe through the folate ligand to FR. These findings confirm that introduction of the fluorophore and palmitoyl groups did not hamper the recognition of the folate moiety against its receptor on the cell.

Fig. 2 Specific binding of the folate-NIR probe to FR (+) KB cells. (A) Specific binding of the synthesized probe 1 to the folate receptor on FR (+) KB cells. (B) Absence of binding of probe 1 to FR (-) A549 cells. The scale bar represents 10 μm .

3.3. *In vivo* tumor-targeted imaging

Mice models bearing FR (+) KB or FR (-) A549 tumors were intravenously injected with 1.5 nmol of probe 2 in the presence or absence of a 100-fold molar excess of free folate.²⁶ Images were acquired at various time points post-injection (p.i.) (Fig. 3). The fluorescence intensity in the tumor as a function of time is plotted in Fig. 3B and D for KB and A549

tumor bearing mice, respectively. As shown in Fig. 3A, FR (+) KB tumor-bearing mice that were injected only with probe 2 initially showed biodistribution of the probe in the whole body and then showed tumor accumulation at 1 h after the injection. After 6 h, probe 2 kept accumulating only in the tumor and liver, while it was cleared from other parts of the body. After 12 h, the accumulation of the probe with a high contrast was only observed in FR-expressing tumors, while the signal in the liver disappeared. However, in FR (+) KB tumor-bearing mice in which the probe was co-injected with an excess amount of free folate, biodistribution and tumor accumulation of the probe were significantly suppressed. As shown in Fig. 3A, the co-presence of an excess amount of free folate largely inhibited specific binding of probe 2 to FR-expressing tumor cells. The probe also showed poor biodistribution in A549 tumor cells lacking FR (Fig. 3C). Quantified data of the probe accumulation in tumor also established the probe specificity *via* the folate ligand-receptor interaction. As shown in Fig. 3B, the tumor-to-normal tissue (T/N) ratio was reduced more than 75% by co-injection of excess folate.

Organ distribution of the probe also indicated the high specificity of the probe to the FR (+) KB tumor (Fig. 4). Three out of four mice in each group were sacrificed 6 h

Fig. 3 *In vivo* study of probe specificity. (A) Whole-body NIR fluorescence imaging of FR (+) KB tumor-bearing mice injected with 1.5 nmol of probe 2 in the absence (upper panel) or presence (lower panel) of 100-fold molar excess of free folate at 30 min, 1, 2, 6, and 12 h p.i. (B) ROI fluorescence intensity of the tumor as a function of time after injection of 1.5 nmol of probe 2 in the absence or presence of 100-fold of an excess amount of free folate. Results are expressed as mean $\pm$ SD ($n = 3$). ** $P < 0.01$, compared with no treatment. (C) Whole-body NIR fluorescence imaging of FR (-) A549 tumor-bearing mice injected with 1.5 nmol of probe 2 in the absence (upper panel) or presence (lower panel) of 100-fold molar excess of free folate at 30 min, 1, 2, 6, and 12 h p.i. (D) ROI fluorescence intensity of the tumor as a function of time after injection of 1.5 nmol of probe 2 in the absence or presence of 100-fold of an excess amount of free folate. Results are expressed as mean $\pm$ SD ($n = 3$). Statistical results were in comparison with no treatment. N.S.: not significant. Arrows in each panel show the liver and the tumor, respectively.

Fig. 4 Representative fluorescence images of dissected KB and A549 tumors and major organs after noninvasive imaging at 6 h p.i. (A) Fluorescence image of dissected organs. B = blood, Li = liver, Lu = lungs, K = kidney, S = spleen, H = heart, T = tumor. KB tumor-bearing mice injected with 1.5 nmol of probe 2 in the absence (upper panel) or presence (middle) of 100-fold molar excess of free folate at 30 min, 1, 2, 6, and 12 h p.i. The lower panel indicates the same image of organs from FR (-) A549 tumor-bearing mice. (B) ROI fluorescence analysis of dissected tumors and major organs after noninvasive imaging at 6 hours p.i. in FR (+) KB tumor-bearing mice in the absence or presence of excess folate. Results are expressed as mean $\pm$ SD ($n = 3$). ** $P < 0.01$, compared with no treatment.

post injection (p.i). Only one mouse was kept until 12 h for imaging. Tumor, organs, and tissues including the liver, lungs, kidneys, spleen, heart, and blood were excised from mice. *Ex vivo* imaging and evaluation of dissected tissues from mice with probe 2 with or without co-injection of free folate revealed that the probe was mainly distributed in the tumor and kidney. A lesser extent of distribution was observed in the liver, and the probe was detected at very low levels in other tissues. Quantification of fluorescence confirmed that in FR-expressing KB tumor-bearing mice, a high intensity of probe fluorescence could

be detected in the tumor, while co-injection of free folate significantly inhibited probe accumulation in the same type of tumor (Fig. 4B).

Our results also showed that in addition to the accumulation of probe 2 in the tumor, probe 2 was also distributed in the kidney and liver. Accumulation of the probe in the liver is possibly due to the large fenestrations of liver's capillary epithelium (size of 100 to 150 nm depending on the animal species), which allows almost unrestricted passage of plasma components to the perisinusoidal space of the liver.^{12,27} The considerable high probe accumulation in the kidneys also

Fig. 5 Biodistribution of probe 2 and probe 3 in time-course imaging. (A) Whole-body NIR fluorescence imaging of FR (+) KB tumor-bearing mice injected with 1.5 nmol of probe 2 (upper panel) or probe 3 (lower panel) 30 min, 1, 2, 6, 18, and 24 h p.i. (B) ROI fluorescence intensity analysis of the whole body of a mouse as a function of time after injection of probe 2 or probe 3 into FR (+) KB tumor-bearing mice. Results are expressed as mean $\pm$ SD ($n = 3$). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, compared to no treatment. Arrows in each panel show the liver and the tumor, respectively.

could have been caused by the presence of high-affinity folate receptors in proximal tube cells of kidneys.^{27–29}

3.4. Effect of the alkyl chain moiety of the probe on blood circulation

In this study, we designed the probe based on the high binding affinity of the free fatty acid to the hydrophobic pockets of HSA. To examine whether the introduced palmitoyl in the probe molecule can serve as a ligand against circulating HSA, 1.5 nmol of probe 2 or probe 3 (without palmitoyl) was injected in FR(+) KB-tumor bearing mice (Fig. 5). Fluorescence images were acquired at 30 min, 1, 2, 6, 18 and 24 h post-injection. Results indicated that probe 2 accumulated and had long retention in the tumor likely through the ligand–receptor interaction. Probe 3 also accumulated in the tumor, but retention and accumulation in the tumor were shorter than that of probe 2 (Fig. 5). Although the long retention of the probe in the tumor may be caused by the effective binding of the folate ligand to its receptor on KB cells, the obtained long circulation also contributes due to the efficient supply of the probe to the tumor while keeping a high concentration of the probe in blood. In fact, the control probe without the palmitoyl group showed poor circulation in mice, with a decreased retention of the fluorescence signal in the tumor compared with probe 2. Together our results suggest that the design of the imaging probe with reversible binding to HSA that was introduced in this study may be an effective tool for cancer imaging.

4. Conclusions

The incorporation of a long fatty acid in the design of folate-targeted fluorescence imaging molecules showed longer blood circulation and bioavailability in mice bearing folate receptor-positive tumors. This result can be explained by the reversible binding of this probe (probe 2) to hydrophobic pockets of endogenous serum albumin. This approach is attractive to generate small fluorescence imaging probes with a better bioavailability and longer blood circulation, which can result in an improved imaging or therapy.

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