Article

Application of HaloTag[®] Technology to *in Vivo* Molecular Imaging Using Protein Probes Labeled by Metallic Radionuclides

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We are developing new protein probes for in vivo radionuclide imaging using HaloTag[®] interchangeable labeling system. The selective and rapid bond formation between the HaloTag protein and HaloTag ligands can be applied to a more sophisticated labeling method for protein probes as a substitute of avidin–biotin interaction. For this purpose, we propose to use HaloTag–fusion proteins, which comprise targeting domain and HaloTag domain. In this study, we synthesized 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)–HaloTag ligands that could chelate radioactive metals useful for in vivo imaging. DOTA–HaloTag ligands were actually labeled with ¹¹¹In efficiently, and the labeled ligands were successfully conjugated to a POH protein, which is a protein probe designed for imaging of hypoxia inducible factor-1 α activity. The obtained ¹¹¹In-labeled HaloTag–POH protein probes actually accumulated in implanted tumors in mice, which were visualized by single photon emission computed tomography (SPECT).

Key Words: HaloTag, HaloTag ligand, Interchangeable labeling system, metal-chelate, *in vivo* imaging, multimodality imaging

1. Introduction

Recently, molecular imaging studies are attracting strong attention and many imaging probes are being developed. Protein probes including antibodies are most important ones among them. But, it is not so easy to synthesize useful protein probes. Protein probes must be labeled without damaging their active sites. Their elements determining their biological activity must be protected from modification during the labeling process. Protein probes must be labeled under mild conditions around room

temperature so that they would not be degenerated. When protein probes were labeled by conventional methods using an activated ester or thioisocyanate (NCS) derivatives, non-selective tagging to lysine residues often occurred and this was likely to damage biological activities of protein probes.

The introduction of avidin–biotin interaction has overcome this problem. This interaction does not damage biological activities of protein probes because their active sites are not modified in this labeling process. Moreover, this interaction can easily bind signal sources to protein probes with very strong affinity.

Recently, another new labeling method using HaloTag[®] protein was reported.^{1, 2)} A HaloTag[®] protein is a modified bacterial haloalkane dehalogenase designed to site-specifically bind to a chloroalkyl ligand (HaloTag[®] ligand) *via* a covalent ester bond that is irreversible under physiological conditions. HaloTag[®]–HaloTag[®] ligand interaction is stronger and rapider than avidin–biotin one.³⁾ Therefore, HaloTag[®] technology can become a more sophisticated labeling method for protein probes as a substitute of avidin–biotin interaction. These excellent features of HaloTag[®] technology have promoted its application to *in vivo* visualization of the localization, translocation, and interactions of intracellular proteins.

Some researchers have already successfully visualized intracellular distribution of proteins by HaloTag[®] protein labeled with fluorescent dyes.^{1, 4)} Using these HaloTag[®] protein labeled with fluorescent dyes, we also recently successfully visualized the activity of hypoxia inducible factor-1 α (HIF-1 α)⁵⁾ that is a transcription factor stabilized under hypoxic conditions.⁶⁾ We have proposed a chimeric protein probe comprising a protein-transduction domain (PTD),⁷⁾ oxygen-dependent degradation domain (ODD)^{8, 9)} and a HaloTag[®] domain. HaloTag[®] ligands conjugated near-infrared dyes (NIRs) were bound to this protein probe (POH).⁶⁾ This protein probes for optical imaging successfully visualized HIF-1 α -positive tumors. Although the probe labeled with NIRs contributed to the observation of hypoxic environments inside tumors, tumors located in only superficial parts of mice could be observed by the optical probe and the evaluation was not quantitative. These optical probes were not suitable for quantitative evaluation and it was not always easy for them to visualize orthotopically transplanted tumors that were usually located in deep areas in the body.

If HaloTag[®] ligands could be conjugated by radioactive compounds, HaloTag[®] protein probes would be used for radionuclide imaging tests such as

Fig. 1 Protein probe using the HaloTag[®] interchangeable labeling system.

positron emission tomography (PET), single photon emission computed tomography (SPECT). Then, deep areas of living bodies would be successfully visualized *in vivo* and precise quantitative evaluation would be possible. But, unfortunately, to our best knowledge, only a few radiolabeled HaloTag[®] protein probes have been reported.¹⁰⁾

Therefore, we aimed to develop protein probes labeled by metallic radionuclides using HaloTag[®] technology to evaluate HIF-1 α activity in this study. We tried to synthesize HaloTag[®] ligand-metal chelator conjugates using 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) because DOTA derivatives can form complexes with many metal cations¹¹⁾ such as the lanthanide series, Y, Ga, In, and Cu. These metallic elements are beneficial because there are many useful radionuclides such as ¹¹¹In, ^{67/68}Ga, ^{62/64}Cu, ¹⁷⁷Lu, ⁹⁰Y.^{12–15)}

After ¹¹¹In-labeled DOTA–HaloTag[®] ligands were obtained, they were bound to POH protein. Then, these labeled compounds were administered to tumor-bearing mice and their accumulation in tumors, in which hypoxic areas would exist, was imaged by using a SPECT/CT scanner dedicated for

Table 1 Property of metal species and their applications

Metal	Property	Application
¹¹¹ In	Gamma-emission (23, 171, and 245 keV)	SPECT
⁶⁷ Ga	Gamma-emission(93, 184, 300, and 393 keV)	SPECT
⁶⁸ Ga	Positron-emission	PET
^{62/64} Cu	Positron-emission	PET
¹⁷⁷ Lu	Gamma (113 and 208 keV) and beta-emission	SPECT Radionuclide therapy
⁹⁰ Y	Beta-emission	Radionuclide therapy
Gd	Paramagnetic effect	MRI
¹⁵⁷ Gd	Thermal neutron capture reaction	Gd-Neutron capture therapy

small animal imaging.

2. Materials and methods

2.1 Reagents and radionuclides

HaloTag® ligand **2** and DOTA derivative **1** were purchased from Promega Corporation (Madison, WI, USA) and Macrocyclics, Inc. (Dallas, TX, USA), respectively. Thin layer chromatography (TLC) plate was purchased from Merck Millipore (Cat. No.: 1.05721.0001, Darmstadt, Germany). Other reagents and solvents were purchased from Sigma-Aldrich Japan (Tokyo, Japan) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan). [¹¹¹In] InCl₃ in 0.04 N HCl solution was provided by Nihon Medi-Physics (Nishinomiya, Japan) based on the collaboration contract between our institute and this company. This contract was approved by our institutional committee of collaboration.

2.2 General procedures for the purification and characterization of ligands

Reverse-phase high-performance liquid chromatography (RP-HPLC) analyses and purification were undertaken on a LaChrom Elite system (Hitachi, Tokyo, Japan) or Prominence UFLC system (Shimadzu, Kyoto, Japan) equipped with a COSMOSIL MS-II (particle size: 5 μm, 4.6×150 mm) analytical column (Nacalai Tesque, Inc., Kyoto, Japan) at a flow rate of 1 mL/min. Liquid chromatography-mass

spectrometry (LC-MS) analyses were performed on a Prominence UFLC (Shimadzu, Kyoto, Japan)–API 3200 (AB SCIEX; Foster City, CA, USA) system. For RP-HPLC and LC-MS analyses, a COSMOSIL MS-II (particle size: 5 μm, 4.6×150 mm) analytical column (Nacalai Tesque, Inc., Kyoto, Japan) was employed at a flow rate of 0.2 mL/min. A solvent system consisting of water/acetonitrile containing 0.1% trifluoroacetic acid was used for HPLC elution. The absorption of the eluate was detected by HPLC-UV at 254 nm. Mass spectra using electron spray ionization (ESI) were obtained from an API 3200 system (AB SCIEX).

2.2.1 Synthesis of 10-[N-(18-chloro-3,6,9,12-tetraoxaoctadecyl)acetamido]-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (DOTA-AMHL) **3**

In this study, HaloTag® (O4) ligand four ethylene glycol linkers **2** was selected, because HaloTag® (O4) has a longer linkage compared with other commercially available HaloTag® ligands. Insertion of longer linker between DOTA and HaloTag® ligands will avoid the interference of the conjugation of HaloTag® ligand to the HaloTag® protein. HaloTag® ligand **2** (0.5 mg, 1.5 μmol) and DOTA derivative **1** (5.9 mg, 7.7 μmol) were dissolved in 200 μL of 10% *N,N*-Diisopropylethylamine (DIPEA) solution in dimethylformamide (DMF). The solution was stirred for 2 h at 60°C. The solvent was concentrated

Fig. 2 Synthesis of DOTA-AMHL 3 and radiosynthesis of $[^{111}\text{In}]\text{In-DOTA-AMHL 4}$.

under reduced pressure. The residue was charged on a pre-charged C18 cartridge (SepPak C18; Waters, Millford, MA, USA) and eluted with water/acetonitrile containing 0.1% trifluoroacetic acid to give DOTA-AMHL 3. Eluted fractions were analyzed by labeling of the aliquots with $[^{111}\text{In}]\text{InCl}_3$ and thin-layer chromatography (TLC). Eluted fractions were also checked by LC-MS instead of ^{111}In -labeling (MS (ESI) m/z : $[\text{M}+\text{H}]^+$ 698, 700).

2.2.2 Radiolabeling of DOTA-AMHL 3 with ^{111}In

To a solution of $[^{111}\text{In}]\text{InCl}_3$ (250.7 MBq) in 1.5 mL of 0.04 N HCl was added 0.3 mL of 0.2 M ammonium acetate solution (pH=5.5) and DOTA-AMHL 3 (4.2 μg , 6.0 nmol) dissolved in 2 μL of DMF. The reaction mixture was heated to 60 °C for 1 h. After filtration with a membrane filter (0.2 μm , Millex; Millipore, Milford, MA, USA), the filtrate was purified by RP-HPLC to afford $[^{111}\text{In}]\text{In-DOTA-AMHL 4}$ in (radiochemical yield: 85%).

2.3 Conjugation of POH to a radiolabeled HaloTag[®] ligand

POH protein was prepared as described in the previous report.⁶⁾ It was then dissolved in Mg^{2+} - and Ca^{2+} -free phosphate-buffered saline (PBS; pH=8.0). ^{111}In -labeled HaloTag[®] ligand (29.0 MBq) dissolved in 80 μL of Mg^{2+} - and Ca^{2+} -free PBS (pH=8.0) was added to 20 μL of 1 M Tris HCl buffer and POH protein (200 μg) dissolved in 100 μL of PBS. The solution was incubated at 4 °C overnight. The unbound 4 was removed by gel filtration using *ca.* 2 g of Sephadex

G50-Fine gel (GE Healthcare, Waukesha, WI, USA) to afford $[^{111}\text{In}]\text{POH-AM}$. The average radiochemical yield in this conjugation reaction was $86 \pm 6\%$, and the protein was quantitatively recovered. These experiments were performed in triplicate. To validate the conjugation of POH with ^{111}In -labeled HaloTag[®] ligand and the purity of this conjugated ligand, samples of labeled protein and unlabeled protein were heat-denatured at 90 °C with 2-mercaptoethanol, and then electrophoresed on 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel (READY GELS J; BioRad, Hercules, CA, USA) and the gels stained by Coomassie Brilliant Blue R-250 staining solution (CBB; BioRad). Subsequent autoradiography (ARG) of the gels was achieved by exposure on an imaging plate that was read with a FLA-7000 imaging analyzer (Fujifilm, Tokyo, Japan). The protein molecular weight markers in ARG were obtained by plotting $[^{111}\text{In}]\text{InCl}_3$ solution on the protein standard markers.

2.4 *In vivo* imaging of POH protein conjugated to ^{111}In -labeled DOTA-HaloTag[®] ligands

POH protein conjugated to ^{111}In -labeled DOTA-HaloTag[®] ligands (18 MBq/100 μg of protein) was intravenously injected from the tail vein of FM3A tumor-bearing C3H mice. SPECT images of mice were acquired 24 h after the injection by a SPECT/CT scanner (NanoSPECT/CT, Bioscan, Washington, D.C., USA). Images were obtained for 70 min and the obtained image data were reconstructed according to a routine protocol.

3. Results

3.1 Synthesis of DOTA–HaloTag® ligands

We synthesized DOTA–HaloTag® ligands: their structures and synthetic routes are summarized in Fig. 2. Amidation of pre-activated *N*-hydroxysuccinimide esters of DOTA **1** with HaloTag® amine (O4) ligand **2** in the presence of DIPEA afforded the desired product DOTA-AMHL **3**.

In the synthesis of DOTA-AMHL **3**, an excess amount of DOTA derivative was used because of

the increase in reaction efficiency. Purification of the reaction mixture of DOTA-AMHL **3** was troublesome because the product **3** and the excess amount of DOTA derivative **1** had no chromophore and could not be detected by UV absorption, though disappearance of the HaloTag® amine **2** was confirmed by staining with ninhydrin that was the reagent for detection of primary amine. Therefore, after separation on a SepPak C18 column, all eluted fractions were collected and their aliquots were labeled with ¹¹¹In, followed by radio-TLC analyses to visualize product **3** and the byproducts derived from DOTA (including **1**) by ARG (Fig. 3). The objective compound, DOTA-AMHL was thought to be eluted in 30% acetonitrile fractions. Then, the fractions were analyzed by mass spectrometry to identify the product. The molecular weights (*m/z*: [M+H]⁺) of the compound containing in the fractions was 698/700 that was identical to the desired product, DOTA-AMHL **3** (Fig. 4).

Fig. 3 TLC analysis of crude products of DOTA-AMHL **3**.

3.2 Radiolabeling of DOTA–HaloTag® ligands

The optimal condition of ¹¹¹In-labeling reaction to maximize the radiochemical yield and prevent the formation of hydroxo complexes of indium was to react at 60°C in 0.2 M ammonium acetate at a pH of 5.5. The crude mixture was purified by RP-HPLC eluted

Fig. 4 Mass spectrum of DOTA-AMHL **3**.

Fig. 5 Radio-HPLC chromatogram of [^{111}In]In-DOTA-AMHL 4.

with acetonitrile/ H_2O containing 0.1% trifluoroacetic acid, and the radio-HPLC chromatogram showed almost one peak with a retention time of 9.0 min and this result indicated the satisfactory purity of this product (Fig. 5). Finally, the radiolabeled products [^{111}In]In-DOTA-AMHL 4 was obtained in 85% yield.

3·3 Conjugation of a HaloTag[®]-fusion protein to a radiolabeled HaloTag[®] ligand

As a final process for the construction of an *in vivo* imaging protein probe, POH protein was conjugated to the ^{111}In -labeled HaloTag[®] ligand. POH protein and the HaloTag[®] ligand 4 were incubated in PBS (pH=8.0) at 4°C. After overnight incubation, [^{111}In]POH-AM was then purified by gel filtration to remove unreacted ligands. The radiolabeling efficiency of [^{111}In]POH-AM were $86 \pm 6\%$ ($n=3$). Protein and radiochemical purity was validated by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 6). A single band of ^{111}In -labeled POH protein was detected at 46 kDa that was identical to that of an unlabeled control POH protein. ARG analysis of the gel also showed the single band, i.e., the POH was conjugated to ^{111}In -labeled HaloTag[®] ligand without protein degradation.

3·4 *In vivo* imaging of POH protein conjugated to ^{111}In -labeled DOTA-HaloTag[®] ligands

SPECT/CT images revealed small amount of

Fig. 6 SDS-PAGE of [^{111}In]POH-AM. (A) POH and [^{111}In]POH-AM resolved by SDS-PAGE were stained with CBB. (B) ARG analysis of the resolved proteins.

Fig. 7 *In vivo* SPECT image of POH protein conjugated to ^{111}In -labeled DOTA-HaloTag[®] ligands. Maximal intensity projection image of MFA tumor implanted around the shoulder region of the mouse →: Activity in the tumor.

[^{111}In]POH-AM protein conjugated to ^{111}In -labeled DOTA-HaloTag[®] ligands accumulated in FM3A tumors implanted in the shoulder regions of C3H mice (Fig. 7).

4. Discussion

In this study, we investigated new protein labeling method for radionuclide imaging by using HaloTag®–HaloTag® ligand system.

We used DOTA as a chelating agent for radioactive metals because it was reported that DOTA can chelate various kinds of metallic elements.

DOTA was successfully combined to HaloTag® ligand and obtained compounds were labeled with ¹¹¹In in good yield. And, these radiolabeled [¹¹¹In] In-DOTA–HaloTag® ligands could be conjugated to a HaloTag® domain on a POH protein, resulting in the successful construction of ¹¹¹In-labeled protein probes for *in vivo* HIF-1 imaging.

Our results demonstrated that HaloTag® technique can be applied to radionuclide imaging in addition to optical imaging previously reported.⁶⁾ Unlike optical imaging, radionuclide imaging method can quantitatively visualize deep areas of living bodies. Therefore, our study would contribute to wide application of HaloTag® technique in the field of molecular imaging.

But, there are some problems in the current HaloTag® radionuclide imaging method. Our SPECT images showed that the obtained signal was too weak. This indicated that the accumulated dose of protein probes labeled by HaloTag® technique was too small to clearly visualize the targets in the living body. We observed that rapid blood clearance of this protein due to immediate excretion from liver and kidney after injection (data not shown). These results suggested that this pharmacokinetics prevent [¹¹¹In] POH-AM from accumulating tumor tissues. Therefore, to facilitate tumor uptake of this hypoxic imaging agent, further modification such as PEGylation to enhance blood retention might be necessary.¹⁶⁾

Although there are some problems to be resolved, we would like to emphasize that this HaloTag® technique is advantageous to easily conjugate various metal chelates to a protein without changes in the

biological activity or affinity.

5. Conclusion

We successfully synthesized ¹¹¹In-labeled radioactive protein probes for the visualization of HIF-1 activity using DOTA–HaloTag® ligands. *In vivo* SPECT images.

Since this methodology can be applied to other metallic signal sources, it would contribute to investigations in the field of multimodal *in vivo* imaging.

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References

- 1) Los, G. V., Encell, L. P., McDougall, M. G., Hartzell, D. D., Karassina, N., Zimprich, C., Wood, M. G., Learish, R., Ohana, R. F., Urh, M., Simpson, D., Mendez, J., Zimmerman, K., Otto, P., Vidugiris, G., Zhu, J., Darzins, A., Klaubert, D. H., Bulleit, R. F. and Wood, K. V., HaloTag: a novel protein labeling technology for cell imaging and protein analysis, *ACS Chem. Biol.*, **3**, 373–382 (2008)
- 2) Los, G. V. and Wood, K., The HaloTag: a novel technology for cell imaging and protein analysis, *Methods Mol. Biol.*, **356**, 195–208 (2007)
- 3) Los, G., Darzins, A., Karassina, N., Zimprich, C., Learish, R., McDougall, M., Encell, L., Friedman-Ohana, R., Wood, M. and Vidugiris, G., HaloTag™ Interchangeable labeling technology for cell imaging and protein capture, *Cell Notes*, **11**, 2–6 (2005)
- 4) Kosaka, N., Ogawa, M., Choyke, P. L., Karassina, N., Corona, C., McDougall, M., Lynch, D. T., Hoyt, C.

- C., Levenson, R. M., Los, G. V. and Kobayashi, H., *In vivo* stable tumor-specific painting in various colors using dehalogenase-based protein-tag fluorescent ligands, *Bioconjug. Chem.*, **20**, 1367–1374 (2009)
- 5) Semenza, G. L., Targeting HIF-1 for cancer therapy, *Nat. Rev. Cancer*, **3**, 721–732 (2003)
- 6) Kuchimaru, T., Kadosono, T., Tanaka, S., Ushiki, T., Hiraoka, M. and Kizaka-Kondoh, S., *In vivo* imaging of HIF-active tumors by an oxygen-dependent degradation protein probe with an interchangeable labeling system, *PLoS ONE*, **5**, e15736 (2010)
- 7) Schwarze, S. R., Ho, A., Vocero-Akbani, A. and Dowdy, S. F., *In vivo* protein transduction: Delivery of a biologically active protein into the mouse, *Science*, **285**, 1569–1572 (1999)
- 8) Harada, H., Hiraoka, M. and Kizaka-Kondoh, S., Antitumor effect of TAT-oxygen-dependent degradation-caspase-3 fusion protein specifically stabilized and activated in hypoxic tumor cells, *Cancer Res.*, **62**, 2013–2018 (2002)
- 9) Harada, H., Kizaka-Kondoh, S. and Hiraoka, M., Mechanism of hypoxia-specific cytotoxicity of procaspase-3 fused with a VHL-mediated protein destruction motif of HIF-1 α containing Pro564, *FEBS Lett.*, **580**, 5718–5722 (2006)
- 10) Hong, H., Benink, H. A., Zhang, Y., Yang, Y., Uyeda, H. T., Engle, J. W., Severin, G. W., McDougall, M. G., Barnhart, T. E., Klaubert, D. H., Nickles, R. J., Fan, F. and Cai, W., HaloTag: a novel reporter gene for positron emission tomography, *Am. J. Transl. Res.*, **3**, 392–403 (2011)
- 11) Liu, S. and Edwards, D. S., Bifunctional chelators for therapeutic lanthanide radiopharmaceuticals, *Bioconjug. Chem.*, **12**, 7–34 (2000)
- 12) De León-Rodríguez, L. M. and Kovacs, Z., The synthesis and chelation chemistry of DOTA–Peptide conjugates, *Bioconjug. Chem.*, **19**, 391–402 (2008)
- 13) Fichna, J. and Janecka, A., Synthesis of target-specific radiolabeled peptides for diagnostic imaging, *Bioconjug. Chem.*, **14**, 3–17 (2002)
- 14) Wadas, T. J., Wong, E. H., Weisman, G. R. and Anderson, C. J., Coordinating radiometals of copper, gallium, indium, yttrium, and zirconium for PET and SPECT imaging of disease, *Chem. Rev.*, **110**, 2858–2902 (2010)
- 15) Wangler, B., Schirmacher, R., Bartenstein, P. and Wangler, C., Chelating agents and their use in radiopharmaceutical sciences, *Mini Rev. Med. Chem.*, **11**, 968–983 (2011)
- 16) Chapman, A. P., PEGylated antibodies and antibody fragments for improved therapy: a review, *Adv. Drug Deliv. Rev.*, **54**, 531–545 (2002)

要 旨

金属放射性核種で標識した蛋白質プローブを用いた HaloTag技術のインビボ分子イメージングへの応用

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我々は腫瘍内低酸素環境を評価するために低酸素誘導因子の活性を測定することを試みているが、HaloTagシステムを利用して、インビボ核医学イメージング用のタンパク質標識プローブの作製を検討した。HaloTagリガンドにキレート剤DOTAを結合させたものを¹¹¹Inで標識し、それをHaloTagタンパク質と結合させて、低酸素誘導因子活性評価用のプローブPOHと組み合わせてプローブを作製した。