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2-Nitroimidazole-Tricarbocyanine Conjugate as a Near-Infrared Fluorescent Probe for *in Vivo* Imaging of Tumor Hypoxia

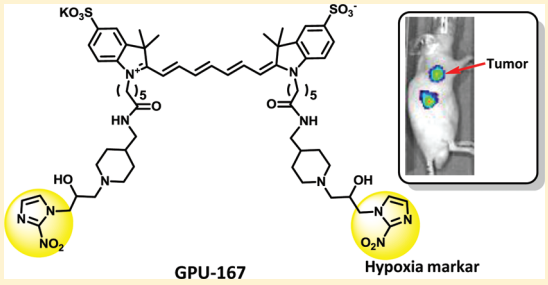
Kensuke Okuda,[†] Yasuyuki Okabe,[†] Tetsuya Kadonosono,[‡] Takahiro Ueno,[†] Bahaa G. M. Youssif,[†] Shinae Kizaka-Kondoh,[‡] and Hideko Nagasawa*,[†]

[†]Laboratory of Pharmaceutical and Medicinal Chemistry, Gifu Pharmaceutical University, Gifu 501-1196, Japan

[‡]Department of Biomolecular Engineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama 226-8501, Japan

Supporting Information

ABSTRACT: We developed a novel near-infrared (NIR) fluorescent probe, GPU-167, for *in vivo* imaging of tumor hypoxia. GPU-167 comprises a tricarbocyanine dye as an NIR fluorophore and two 2-nitroimidazole moieties as exogenous hypoxia markers that undergo bioreductive activation and then selective entrapment in hypoxic cells. After treatment with GPU-167, tumor cells contained significantly higher levels of fluorescence in hypoxia than in normoxia. *In vivo* fluorescence imaging specifically detected GPU-167 in tumors 24 h after administration. *Ex vivo* analysis revealed that fluorescence showed a strong correlation with hypoxia inducible factor (HIF)-1 active hypoxic regions. These data suggest that GPU-167 is a promising *in vivo* optical imaging probe for tumor hypoxia.



The microenvironments of solid tumors are characterized by low pO_2 and pH, which are considerably below physiological levels and completely different from those of normal tissues.[1] The hypoxic status of various solid tumors has been considered to be an indicator of adverse prognosis because of tumor progression toward a more malignant phenotype with increased metastatic potential and resistance to treatment.[2] Tumor hypoxia should be measured to assess the aggressiveness of the tumor and predict the outcome of therapy. Therefore, over the past decade, various methods have been employed to assess the level of oxygenation in solid tumors, including invasive and noninvasive techniques using various modalities, such as positron emission tomography (PET), single-photon emission computed tomography (SPECT), and magnetic resonance imaging (MRI). Various exogenous and endogenous markers for hypoxia are also currently available and have been studied in relation to each other for the evaluation of tumor microenvironments.[3,4]

Recently, more studies have been conducted using noninvasive optical imaging because it is safer and easier to perform than nuclear imaging.[5] Several small molecule fluorescent probes have been developed for hypoxia.[6−10] They rely on the change in fluorescence properties after selective biological reduction in tumor hypoxic regions. However, these fluorescent probes have some drawbacks. The greatest drawback is that they rely on chromophores with maximum absorption/fluorescence peaks in the ultraviolet/visible region of the spectra. Because of insufficient tissue penetration into the region of interest and absorption by endogenous biomolecules (e.g., hemoglobin), most excitation (Ex)/emission (Em) light could not be utilized for detection, causing inadequate *in vivo* imaging. Autofluorescence refers to the intrinsic fluorescence of the tissue caused by excitation by optical radiation, and it can hamper *in vivo* imaging because of poor signal-to-noise ratios. To overcome these drawbacks and improve noninvasive *in vivo* fluorescent imaging, the use of light in the near-infrared (NIR) region (700−900 nm) is a promising alternative. Light scattering and autofluorescence are decreased and tissue penetration is increased in NIR, thereby leading us to attempt to develop an NIR probe that provides a high signal-to-background ratio *in vivo*. The optical imaging is currently mechanistically limited to use in superficial tumors because of the minimal tissue penetration (<1−2 cm depth) even by NIR wavelength light. However, NIR fluorescence cancer imaging is a growing field for preclinical and clinical applications due to its advantages such as high spatial resolution, portability, and real-time display.[11] Actually, NIR fluorescence probes are being applied to endoscopy and surgical guidance in humans despite its limited penetration depth. Accordingly, NIR fluorescence imaging could be one of the powerful tools for future seamless transition between research and clinical cancer imaging technology.

Recently, a hypoxia probe with Ex/Em wavelengths of 650/670 nm, which are in the visible-NIR transition region, was developed by Nagano et al.[12] This was the first report of *in vivo* fluorescence imaging for acute ischemia hypoxia. *In vivo*

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imaging of tumors transplanted from the skin surface of mice has also been achieved with Ex/Em wavelengths of 605/645 nm using a novel indium complex as a hypoxia-sensing phosphorescence probe.[13] Here we report the development of a hypoxia NIR fluorescent probe, GPU-167, with longer Ex/Em wavelengths of 753/778 nm than those used in previous studies, and the evaluation of GPU-167 for the *in vivo* imaging of tumor hypoxia using mouse xenograft models.

In the development of imaging probes for tumor hypoxia, nitroimidazoles have received particular attention as exogenous markers because of their unique behavior in hypoxic environments owing to their high electron affinity.[4,14] The specificity of nitroimidazole imaging would depend on oxygen concentration as well as nitroreductase levels in the tumor cells. Nitroaromatics are selectively reduced by nitroreductase enzymes under hypoxic conditions to form reactive products that can irreversibly bind to cellular nucleophiles (Figure 1). Currently,

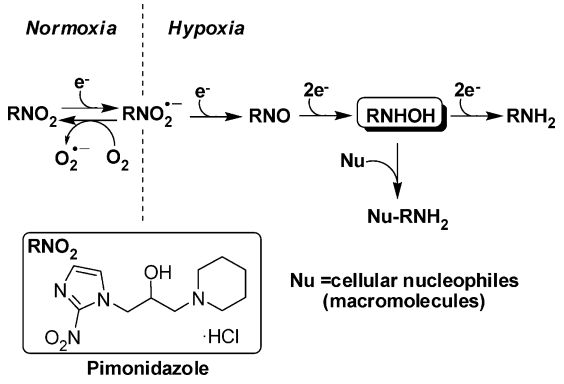

Figure 1. Proposed mechanism for hypoxia selective covalent binding reaction of hydroxylamine intermediates provided by bioreductive metabolism in hypoxia with cellular nucleophiles.

Pimonidazole (Pimo) is believed to be the standard exogenous hypoxic marker for immunohistochemical assessment of hypoxia in solid tumors. The binding of nitroimidazole hypoxia markers such as Pimo to cellular macromolecules has been shown to increase considerably below an oxygen concentration of 10 mmHg and is believed to indicate chronic hypoxia.[15] Recently, a 2-nitroimidazole-indocyanine green (ICG) conjugate was reported to persist in the tumors for a significantly longer period than ICG as shown by NIR-fluorescence tomography,[16,17] suggesting a possible application of the hypoxia selectivity of 2-nitroimidazole for *in vivo* fluorescence imaging. Although the author confirmed existence of hypoxia in tumor using anti-Pimo antibody stain after sacrifice, it was difficult to correlate the NIR fluorescence image with the Pimo staining.[17] Therefore, we designed GPU-167, a novel functionalized tricarbocyanine NIR fluorescence dye conjugated with a Pimo moiety for noninvasive detection of hypoxia in tumors and evaluated the potency and function of GPU-167 as an *in vivo* hypoxia marker by comparison to bioluminescent imaging of hypoxia inducible factor (HIF)-1 expression using a mouse xenograft model of SUIT-2/HRE-Luc cells.[18]

GPU-167 is composed of tricarbocyanine dye as a NIR fluorophore and two Pimo moieties as hypoxia markers with an alkyl linker to combine the two Pimo moieties with the tricarbocyanine dye and synthesized as shown in Scheme 1. Because of the synthetic convenience saving extra synthetic steps, we designed symmetric cyanine dye which contained dual Pimo moiety. The process of synthesis was as follows. First, 4-aminomethylpiperidine (**1**) was converted to Schiff base *N*-benzylidene-1-(piperidin-4-yl)methanamine (**2**),[19] then coupled to 2-nitro-1-(2-oxiranylmethyl)-1*H*-imidazole (**3**)[20,21] to yield 1-[4-(aminomethyl)piperidin-1-yl]-3-(2-nitro-1*H*-imidazol-1-yl)propan-2-ol hydrochloride (**4**) in 61% yield (3 steps), which was a derivative of Pimo with a functional amino group to be covalently modified. Tricarbocyanine dye **6**, which possesses bis alkyl linker moieties with carboxyl groups, was prepared from 1-(ε-carboxypentyl)-2,3,3-trimethyl-3*H*-indolenium-5-sulfonate (**5**) as previously reported.[22] After activation of the carboxylic groups by disuccinimidyl carbonate, reaction with compound **4** yielded the final target molecule GPU-167. It was purified by C18 reversed-phase flash chromatography. Purity and identity were determined by LCMS-IT to yield a single peak and a high-resolution mass spectrum with m/z 1299.5897 for $C_{63}H_{87}N_{12}O_{14}S_2$ ([M-K +2H]$^+$) (see Supporting Information). The evaluation of photophysical properties of GPU-167 was revealed to show narrow absorption and emission bands between the absorption and emission peaks at 753 and 778 nm respectively, which are typical wavelengths of the tricarobocyanine dye and in the NIR region of the spectrum (Figure 2). Its large extinction coefficient (4.8×10^4 M^{-1} cm^{-1} at 753 nm), high quantum yield (0.18), and sufficient water solubility showed that GPU-167 had adequate optical properties to be used as a fluorescent probe for *in vivo* imaging.

First, to evaluate the specific binding of GPU-167 to hypoxic cells, an *in vitro* fluorescence assay was performed with SUIT-2/HRE-Luc pancreatic cancer cells, which stably carry plasmid p5HRE-luciferase and express firefly luciferase under the influence of a HIF-1-dependent promoter.[18] The cells were preincubated under normoxic (21% O_2) or hypoxic (0.1% O_2) conditions for 4 h, followed by incubation with 0.2−20 μM of GPU-167 for 30 min. After the cells were washed, they were further incubated in fresh medium for 1 h under aerobic conditions to release unbound probe. The fluorescence intensity of the cell suspension was measured in a radioimmunoprecipitation assay (RIPA) buffer with excitation at 740 nm and emission at 780 nm. As shown in Figure 3, treatment with GPU-167 at concentrations of 5 and 20 μM resulted in the detection of significantly higher levels of fluorescence in the cells cultured under hypoxia than those in cells cultured under normoxia. These results indicated that GPU-167 might bind to intracellular substances irreversibly and be selectively retained in hypoxic cells for more than 48 h. However, fluorescence intensity under normoxia was also rather high because the probe may cause nonspecific noncovalent binding to cell components and/or persisting as an unbound form in cells due to its electronic and lipophilic features.

Next, *in vivo* imaging of hypoxic cells in tumor-bearing mice inoculated with SUIT-2/HRE-Luc cells was attempted.[23] The SUIT-2/HRE-Luc cells stably carry 5HRE-luciferase,[18] a luciferase reporter gene under the control of the HIF-1-dependent promoter, and thus HIF-1-activity in xenografts can be monitored by bioluminescence imaging. All of fluorescence images were acquired with IVIS imaging system using a 710 ± 15 nm the excitation filter and an 800 ± 10 nm emission filter. When GPU-167 was injected at a dose of 10 nmol into mice carrying subcutaneous xenografts of SUIT-2/HRE-Luc cells via the tail vein, fluorescence signals were detected throughout the body of the mice within 30 min and gradually became weaker (Figure 4A). Signals of GPU-167 in tumors were detected as

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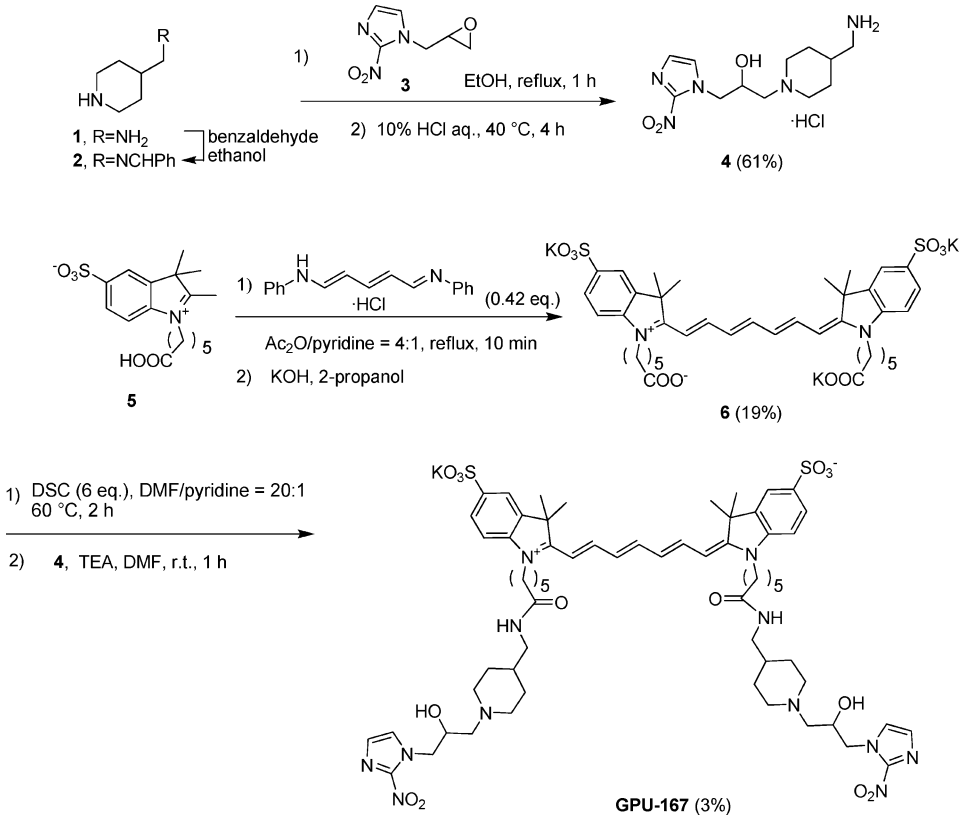
Scheme 1. Synthesis of Hypoxia Probe, GPU-167



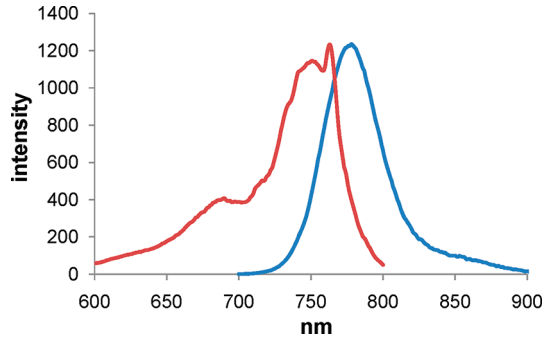


Figure 2. Excitation (magenta) and emission spectra (blue) of GPU-167 in MeOH.

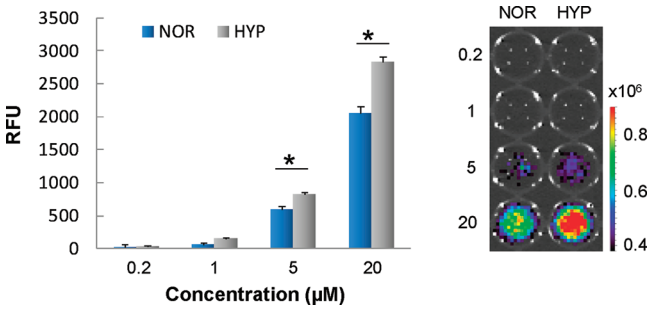


Figure 3. Specific binding of GPU-167 to hypoxic cells *in vitro*. The SUIT-2/HRE-Luc cells (2×10^5 cells) cultured under normoxic (NOR) or hypoxic (HYP) conditions were treated with GPU-167 and analyzed by measurement of fluorescent intensity with an Ex/Em wavelength of 740 nm/780 nm ($^*P < 0.05$, $n = 3$). Representative fluorescence images are also shown in right.

soon as 3 h after injection (Figure 4A). Most of the GPU-167 was rapidly cleared from the mice within 3 h (Figure 4B), and tumor-specific images were obtained 24 h after GPU-167 administration, resulting in the highest T/B ratio (Figure 4C). In addition to the clear accumulation of GPU-167 in the tumors, fluorescence signal was also detected in the abdominal region (Figure 4A). Then, to determine whether GPU-167 accumulation actually correlated with hypoxic conditions within the tumors, a bioluminescent signal emitted from HIF-1-active hypoxic cells in SUIT-2/HRE-Luc xenografts 24 h after administration of GPU-167 was also examined (Figure 4A, right panel). A positive correlation was observed between the fluorescent and luminescent images of the tumor, suggesting that the fluorescence owing to GPU-167 in the tumor was related to hypoxic conditions.

For a more detailed examination, we performed *ex vivo* imaging. Although the comparison of fluorescence by GPU-167 with immunohistochemical staining by anti-Pimo antibody of a tissue section might be helpful for clarifying their spatial relationship, such a double stain analysis needs cumbersome and difficult experiments. On the other hand, the SUIT-2/HRE-Luc xenograft is superior for detection hypoxic tumor cells *in vivo* and *ex vivo* directly. High fluorescence intensity for GPU-167 was detected in the liver, and a moderate intensity was detected in the tumors, kidney, and stomach (Figure 5A,B). In the SUIT-2/HRE-Luc tumor cells, HIF-1α expression was undetectable under normoxic and severe hypoxic (pO_2 < 10 mmHg) conditions but significantly increased under relatively mild hypoxic conditions.[18] Therefore, bioluminescence signals from the SUIT-2/HRE-Luc xenografts indicated that the HIF-1-active cells were located in relatively mild hypoxic regions, which proportionally exist adjacent to severe hypoxic regions.[24]

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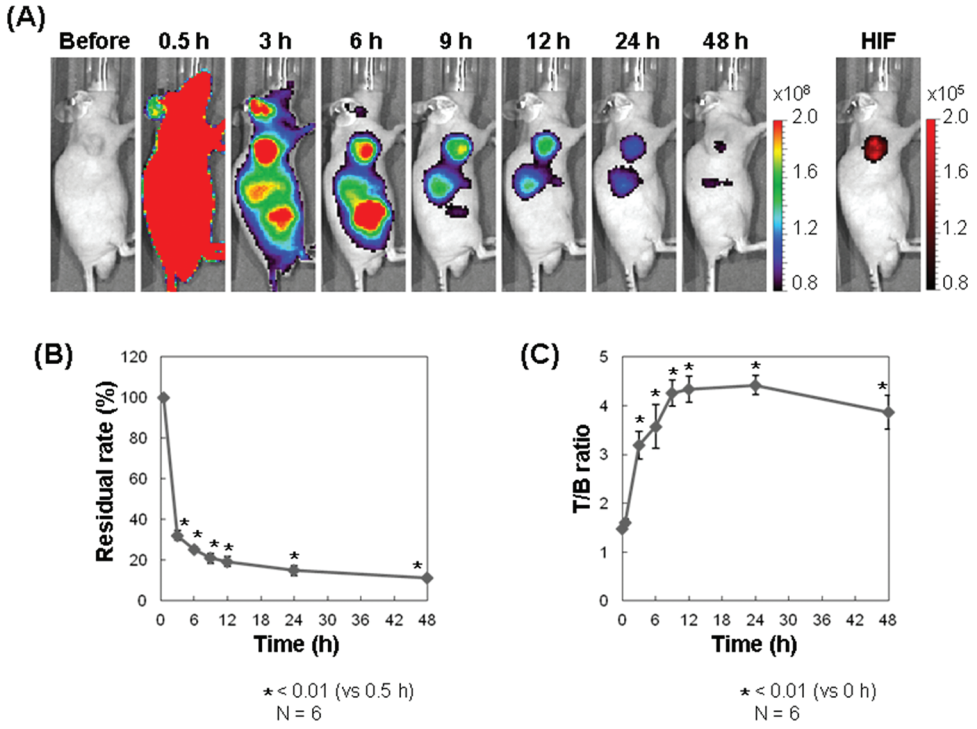

Figure 4. (A) *In vivo* optical imaging of subcutaneous cancers with GPU-167. Representative *in vivo* image after 10 nmol of GPU-167 administration. Nude mice carrying SUIT-2/HRE-Luc xenografts in both forefeet were imaged at the indicated time after GPU-167 injection. The right panel (HIF) shows bioluminescence image of HIF-1-active hypoxic tumor at 24 h. (B) Time-dependent clearance of GPU-167 from whole mouse. Fluorescence intensities of the muscle of the hind foot were measured at the indicated time after administration of GPU-167 and decrease in the intensity was represented by the relative value (Fluorescence intensity at 0.5 h after GPU-167 administration = 100%). (C) Specific accumulation of GPU-167 to subcutaneous xenografts. The relative fluorescence intensity of the tumor to the muscle (T/B ratio) was shown. Fluorescence intensities of the SUIT-2/HRE-Luc xenografts and the muscle of the hind foot were measured at the indicated time after GPU-167 administration. Three mice and six tumors were used for each point on each curve.

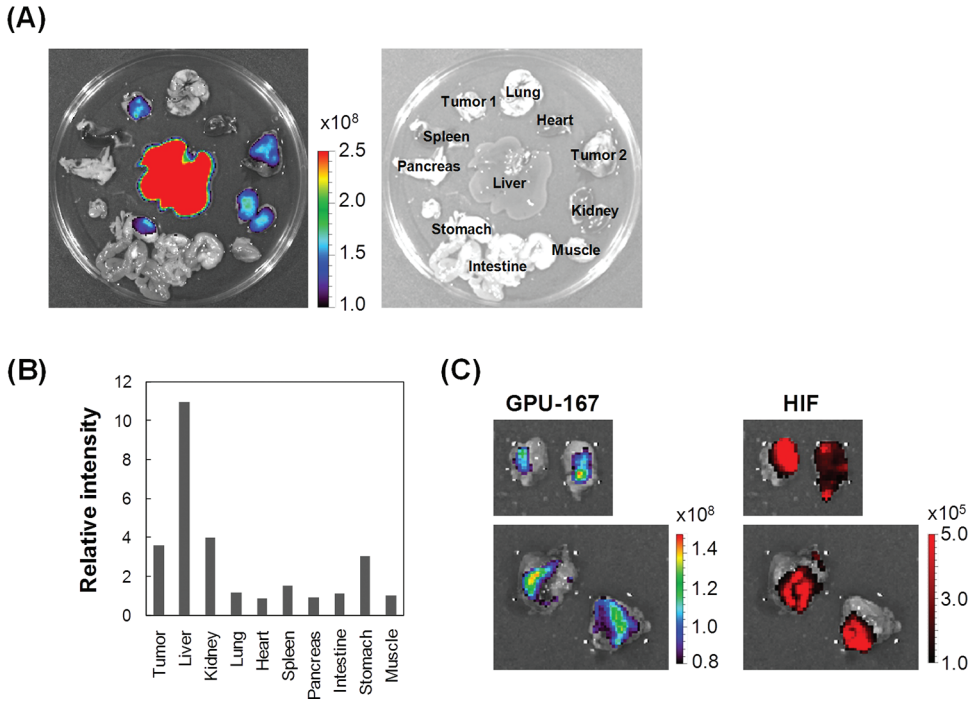

Figure 5. (A) *Ex vivo* fluorescence imaging of organs of tumor bearing mouse. The mouse shown in Figure 4A was analyzed by *ex vivo* imaging. Fluorescence image at 48 h after GPU-167 administration was shown. (B) Fluorescence intensity/organ size values were shown as relative intensity (the value of muscle = 1). (C) *Ex vivo* optical imaging of excised subcutaneous tumors. *Ex vivo* fluorescence (GPU-167) and bioluminescence (HIF) images of excised subcutaneous tumors shown in (A) were analyzed.

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Nitroimidazole such as Pimonidazole compounds are detected at absolute oxygen concentrations below 10 mmHg, suggesting that nitroimidazole probes are specific markers for severe hypoxia. Although these hypoxia markers depend on different mechanisms, their relationship and comparison have been well-researched in various solid tumors.[4] Our immunohistochemical analysis also revealed that the cells with HIF-1α expression and the ones with Pimonidazole-binding were adjacent to each other in SUIT-2/HRE-Luc xenografts.[18] *Ex vivo* imaging of tumors at 48 h after GPU-167 injection revealed that most fluorescence images of GPU-167 were proportional and similar to the bioluminescent images (Figure 5C). These observations suggest that GPU-167 efficiently would detect hypoxic regions *in vivo*. Assessment of such severe hypoxic fractions in tumors, which are regarded not only as adverse prognostic factors but also as indicators of the necessity for tumor-specific treatment, would provide diagnostic and therapeutic advantages for cancer treatment.[25]

Important criteria for effective fluorescence imaging probes include suitable pharmacological properties such as aqueous solubility, low nonspecific binding, rapid clearance of the free dye, and low toxicity that include appropriate photophysical properties such as excitation and emission maxima in the NIR region between 700−900 nm, high quantum yield, and chemical and optical stability. With regard to pharmacological properties of GPU-167, its aqueous solubility was sufficiently high and its hydrophobicity was higher than that of ICG based on its larger hydrophobicity index (Rm), which was 1.62 versus 0.84 for ICG, both determined using a reversed-phase thin layer chromatographic system (mobile phase: 20% 5 mM sodium phosphate buffer (pH 7.4)/80% MeOH). ICG was selectively distributed to the liver and then excreted rather rapidly into the bile, because it was tightly bound to serum proteins[26] and rapidly washed out from tumors in merely less than 3 h.[17] On the other hand, GPU-167 was retained for a relatively longer period (more than 48 h) in the body even though it maintained a good T/B ratio (>2.5). Furthermore, the considerably high liver distribution of GPU-167 disturbed its selective imaging of tumors. Thus, to improve pharmacokinetic properties of GPU-167, further studies of structure−property relationship of NIR probes are required, controlling the number of negatively charged groups (e.g., sulfonates),[27] positively charged groups (e.g., amines), and hydrophobic alkyl groups.

In conclusion, an efficient NIR fluorescent probe, GPU-167, comprising 2-nitroimidazole and tricarbocyanine dye, was developed for *in vivo* imaging of tumor hypoxia. It was trapped in tumor cells cultured under hypoxic condition significantly more than normoxic condition. We also demonstrated that tumors were clearly visualized by NIR fluorescence with significant T/B ratio 24 h after GPU-167 administration to living mice in a mouse xenograft model. We are currently making a structural modification of the hypoxia-selective NIR probe having improved pharmacological properties.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis, purification, and characterization of organic compounds; optical properties; *in vitro* and *in vivo* evaluation. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone and FAX: +81-58-230-8112; E-mail: hnagasawa@gifu-pu.ac.jp.

Notes

The authors declare no competing financial interest.

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