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Abstract. Photoacoustic imaging (PA imaging or PAI) has shown great promise in the detection and monitoring of cancer. Although nanocarrier-based contrast agents have been studied for use in PAI, small molecule contrast agents are required due to their ease of preparation, cost-effectiveness, and low toxicity. Here, we evaluated the usefulness of a novel cyanine dye IC7-1-Bu as a PAI contrast agent without conjugated targeting moieties for *in vivo* tumor imaging in a mice model. Basic PA characteristics of IC7-1-Bu were compared with indocyanine green (ICG), a Food and Drug Administration approved dye, in an aqueous solution. We evaluated the tumor accumulation profile of IC7-1-Bu and ICG by *in vivo* fluorescence imaging. *In vivo* PAI was then performed with a photoacoustic tomography system 24 and 48 h after intravenous injection of IC7-1-Bu into tumor bearing mice. IC7-1-Bu showed about a 2.3-fold higher PA signal in aqueous solution compared with that of ICG. Unlike ICG, IC7-1-Bu showed high tumor fluorescence after intravenous injection. *In vivo* PAI provided a tumor to background PA signal ratio of approximately 2.5 after intravenous injection of IC7-1-Bu. These results indicate that IC7-1-Bu is a promising PAI contrast agent for cancer imaging without conjugation of targeting moieties. © 2014 Society of Photo-Optical Instrumentation Engineers (SPIE)
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Since cancer is one of the leading causes of death worldwide,[1] noninvasive imaging methods for cancer diagnosis are highly valuable. Among several molecular imaging methods, photoacoustic imaging (PA imaging or PAI) has shown great promise in detecting and monitoring cancer since it enables real-time noninvasive imaging of tissues of interest with high contrast but at depths of up to 5 cm.[2,3] PAI can detect not only endogenous biological molecules such as oxyheme and deoxyheme for the assessment of blood flow and oxygen concentration in cancer vasculature,[4] but also exogenous contrast agents targeted to a specific molecular marker of interest to evaluate functional alterations in disease sites.[5] Background signals in PAI can be minimized by using PAI contrast agents that absorb photons at a wavelength of 700–1000 nm [near infrared (NIR) region] in a similar way as that seen for optical imaging (OI) with NIR fluorescence probes.[6,7] Nanosize probes, including iron oxide nanoparticles, carbon nanotubes, gold nanocages, gold nanorods, and nanospheres, which are occasionally conjugated with monoclonal antibodies such as trastuzumab, have been developed as PAI contrast agents[8,9] in order to achieve high probe accumulation into tumor tissues by the enhanced permeability and retention effect. High levels of probe accumulation would be required for PAI because of the relatively low intrinsic sensitivity of this technique compared to OI.[3,10] Although these nanotechnology-based contrast agents have shown their usefulness in PAI, some difficulties in preparation as well as uniformity control, cost-effectiveness, and toxicity are issues that remain to be addressed before these compounds can be used in clinical applications. Considering the potential drawbacks of nanoprobes, small molecule-based contrast agents that could be synthesized through ordinary synthetic procedures would be highly valuable for use in clinical PAI.

We recently developed a novel NIR fluorescence cyanine dye, IC7-1-Bu [3-butyl-2-(2-{3-[2-(3-butyl-1,1-dimethyl-1,3-dihydro-benz[*e*]indol-2-ylidene)-ethylidene]-2-chloro-cyclohex-1-enyl}-vinyl)-1,1-dimethyl-1*H*-benz[*e*]indolium, λex = 823 nm, λem = 845 nm, molecular weight = 667, Fig. 1(a)], which showed unique properties as an OI probe for cancer imaging.[11] IC7-1-Bu accumulated in tumors of living mice after intravenous administration to levels that allowed tumor imaging with OI techniques without conjugation of any tumor-targeting moieties such as monoclonal antibodies or nanocarriers. Building on these previous results, we focus here on the unique tumor-targeting ability of IC7-1-Bu using serum albumin as a drug delivery carrier,[11] and evaluated the potential of IC7-1-Bu as a PAI contrast agent in preclinical experiments using tumor bearing mice. Overall, we obtained additional evidence to support the promising applications of IC7-1-Bu as a PAI contrast agent for tumor imaging in preclinical settings.

IC7-1-Bu was synthesized in three steps from cyclohexanone and 1,1,2-trimethyl-1*H*-benz[*e*]indole as previously reported (the overall yield was 47%). The compound was confirmed by NMR and mass spectrometry.[11] At the beginning of the evaluation, we measured the PA signal of IC7-1-Bu *in vitro* and compared it with that of indocyanine green (ICG), a Food and Drug Administration approved cyanine dye that has been recently applied for PAI after conjugation with a targeting moiety.[12] Specifically, PA signals of IC7-1-Bu and ICG were measured in an aqueous buffer containing 5 g/dL bovine serum albumin with excitation wavelengths of 830 and 810 nm for IC7-1-Bu and ICG, respectively. The wavelengths were selected based on the absorption peaks of each dye. Measured PA signals were then standardized by irradiated laser intensity. As seen in Fig. 1(b), which illustrates the correlation of PA signals
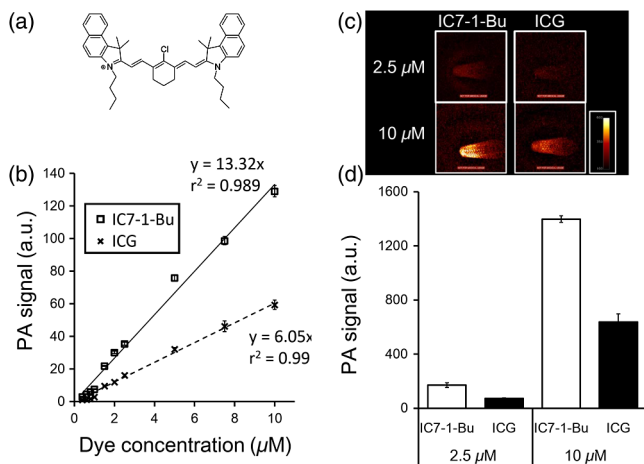
Fig. 1 *In vitro* characterization of IC7-1-Bu as a PAI contrast agent compared to indocyanine green (ICG). (a) The structure of IC7-1-Bu. (b) *In vitro* PA signals of aqueous solutions including IC7-1-Bu or ICG. Correlation between a dye concentration and a PA signal is designated as a line with a zero intercept (solid and dashed lines represent the correlation of PA signals with IC7-1-Bu and ICG, respectively). (c) Representative *in vitro* PA images of solutions including IC7-1-Bu or ICG. (d) *In vitro* PA signals calculated from PA images shown in (c) using AMIDE analysis software.

with the dye concentration in the cuvettes, IC7-1-Bu showed higher PA signals than ICG. The slope of a fitted first-order line with an intercept of zero was approximately 2.2-fold higher for IC7-1-Bu than for ICG. As a next step in the *in vitro* evaluation, we performed PAI of buffer solutions including the dyes (2.5 or 10 μM) using an Endra Life Sciences Nexus 128 instrument (Endra Inc., Ann Arbor, Michigan).[13] The IC7-1-Bu concentration was set to 2.5 μM based on the assumed IC7-1-Bu concentration that accumulated in tumor tissue after intravenous administration as determined from *in vivo* experiments described below. IC7-1-Bu also exhibited a brighter signal with ROI analyses showing that IC7-1-Bu had about a 2.3-fold higher PA signal than that of ICG.

Since these *in vitro* results suggested the potential of IC7-1-Bu as a PAI contrast agent, we next performed *in vivo* PAI experiments using IC7-1-Bu in tumor bearing mice. Animal experiments were conducted in accordance with institutional guidelines and were approved by the Kyoto University Animal Care Committee. Female nude mice (BALB/c nu/nu 4 weeks old), supplied by Japan SLC, Inc., Hamamatsu, Shizuoka, Japan, were housed under a 12-h light/12-h dark cycle and given free access to food (D10001) and water. HeLa cells (2×10^6 cells in 100 μL of phosphate buffered saline, ATCC) were subcutaneously inoculated into the right hind legs of mice. Fourteen days after transplantation, mice were used for the imaging study (the average tumor size was 98 ± 17 mm^3). In an *in vivo* imaging experiment, the Endra Life Sciences Nexus 128 and Clairvivo OPT (Shimadzu Co., Kyoto, Japan)[6,11] were used for PAI and OI, respectively. The whole body OI of tumor bearing mice ($n = 3$ each) revealed strong fluorescence in tumors 24 and 48 h after injection of IC7-1-Bu (0.5 μmol/kg) [Fig. 2(a)], which is in agreement with our previous results.[11] However, ICG (0.5 μmol/kg) showed a rapid clearance of fluorescence via the liver to the intestine [Fig. 2(a)], as would be expected from the reported short biological half-life of ICG.[14] This result clearly suggests that ICG could be used as a PAI contrast agent for tumor detection only after the conjugation of targeting moieties such as monoclonal antibodies. The tumor to background fluorescence ratio, which was calculated by defining fluorescence in the neck area as the background,[6,11] was approximately 2.4 in the mice that were administered IC7-1-Bu 24 or 48 hours earlier. Meanwhile, we further performed *in vivo* PAI with IC7-1-Bu (1.25 μmol/kg) administered to tumor bearing mice ($n = 3$) with an excitation wavelength of 830 nm. We obtained PA images of the tumor region and contralateral muscle region due to the limited field of view of the Nexus 128 system.[13] *In vivo* PAI also showed higher PA signals in tumors 24 and 48 h after intravenous injection of IC7-1-Bu as compared to the preadministration images [Figs. 2(b) and 2(c)]. The tumor to background PA signal ratio calculated by defining PA signals around the contralateral site (left hind leg) as the background
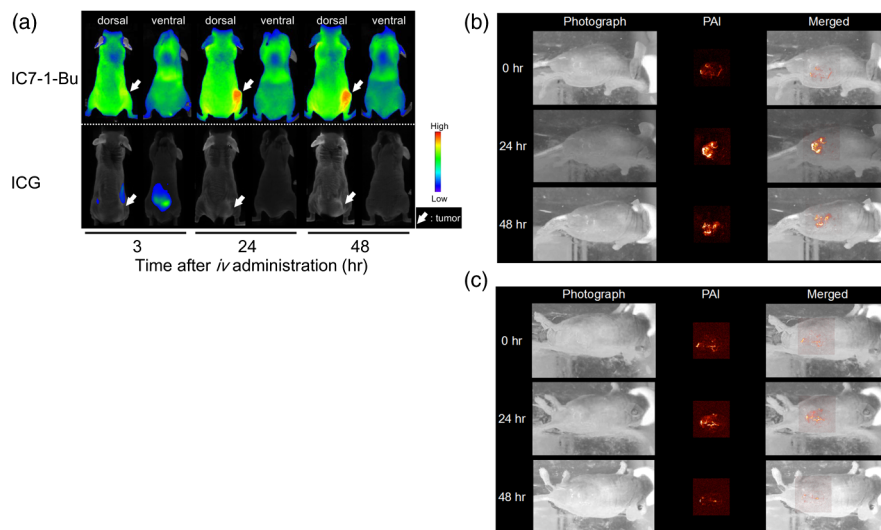

Fig. 2 *In vivo* OI and PAI experiments. (a) *In vivo* OI of tumor bearing mice 3, 24, and 48 h after intravenous injection of IC7-1-Bu and ICG. (b), (c) *In vivo* PAI of tumor bearing mice before administration and at 24 or 48 h after intravenous injection of IC7-1-Bu. Representative PA images of the tumor region (b) and contralateral muscle region (c) are shown in the middle column with photographs and PAI merged photographs in the left and right columns, respectively.

was 1.0 ± 0.1, 3.0 ± 0.3, and 2.3 ± 0.9 at 0, 24, and 48 h, respectively, after intravenous injection of IC7-1-Bu. Finally, we estimated the quantity of IC7-1-Bu accumulated in the tumor 24 h after IC7-1-Bu injection to support the IC7-1-Bu PA signals obtained in the *in vivo* study. To do this, we measured the IC7-1-Bu fluorescence of tumor homogenates ($n = 3$) with Clairvivo OPT and calculated the IC7-1-Bu quantity using a standard curve we prepared separately using known standards. The estimate yielded an uptake rate of $10.0 \pm 0.3\%$ injected dose per gram tissue in tumor, indicating the high tumor targeting ability of IC7-1-Bu. Other tumor targeting probes such as a HER2-targeting liposome and the HER2-targeting monoclonal antibody, trastuzumab, showed about 8%[15] and 20% injected dose per gram tissue,[16] respectively. Therefore, these results strongly support the *in vivo* PAI results described above.

In conclusion, these results indicate that IC7-1-Bu is a promising PAI contrast agent for cancer imaging that does not require conjugation of targeting moieties. Further *in vivo* experiments using varied tumor models are warranted to characterize additional properties of this compound.

Acknowledgments

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Biographies of the authors are not provided.