Supplemental Methods

Materials

All chemicals were obtained from commercial sources and were used without further purification. $^{68}GaCl_4^-$ was eluted from a $^{68}Ge/^{68}Ga$ generator (ANSTO, Lucas Heights, NSW, Australia) with 5 – 8 mL 0.3 M HCl, absorbed and purified on a cationic cartridge, then ^{68}Ga was eluted with 0.5 mL KOH (0.5 M) for radiolabeling. $^{67}GaCl_3$ solution was purchased Nordion (Ottawa, ON, Canada). Radioactivity was measured with a WIZARD™ 3" 1480 γ-counter (PerkinElmer, Waltham, MA) or a dose calibrator (CAPINTEC® CRC-30BC, Ramsey, NJ). Peptide synthesis was performed on a PS3™ automatic peptide synthesizer from Protein Technologies, Inc. (Tucson, AZ). A HPLC system equipped with Shimadzu LC-20 AB binary pumps, SPD-20A Prominence UV/VIS detector, and a BioScan Flow Count radiodetector was used for preparation and analysis of synthesized peptides. UPLC-MS was performed on a Waters system with PDA, MicroMass ZQ and diode assay detector, together with a waters Acquity BEH C18 column (130 Å, 2.1 × 100 mm). The prostate cancer cell line PC-3 was obtained from ATCC (Manassas, VA) and cultured in F-12K medium supplemented with 10% FBS, 1.5 g/L sodium bicarbonate and 2 mM L-glutamine.

PET/CT imaging

Animals were under anesthesia with 2 % isoflurane during PET/CT imaging. Mice were placed in the Inveon PET/CT system with the tumors centered in the field of view at 60 min post injection. After 10 min of data acquisition on PET scanner, the animal was moved to CT scanner. CT acquisition was performed for 4 min at 60 kVp and 0.8 mA with 2 mm aluminum filtration. PET images were reconstructed by 2D-ordered subsets expectation maximization (2D-OSEM) on the Inveon PET/CT system. The reconstructed data of PET and CT images were rendered in 3D or 2D using Inveon Research Workstation (Siemens, Malvern, PA). The calibration factor of the Inveon PET/CT scanner was measured with a mouse-size phantom composed of a cylinder uniformly filled with an aqueous solution of ^{18}F with a known activity concentration. Region-of-interest (ROI) analysis of the acquired images was performed using ASIPro software (Siemens, Malvern, PA), and the observed maximum pixel value (%ID/mL) was utilized to diminish partial volume effects on tumor uptake.

Optical Imaging

With the animals under continuous isoflurane anesthesia, fluorescence images were acquired using multi-filter acquisition mode from 680 – 780 nm (20 nm increment per interval) after excitation with wavelengths of 640 nm, and the acquisition time varied from 1 to 9 s (FOV13.4 and f2). Data were acquired and analyzed using Living Image 4.6 software (Caliper Life

Sciences). For ex-vivo imaging, PC-3 cancer and muscle tissues were collected and placed on a Petri dish for imaging with the same imaging protocol as for in vivo imaging.

For quantitative analysis regions of interest (ROIs) were drawn around the organs and tumors on the white light images. The intensity of the fluorescent signal within the ROI was expressed as the average radiant efficiency p/s/cm^2/sr (number of photons (p) per second (s) per surface area (cm^2) per steradian (sr)) per μW/cm^2. Semiquantitative analysis of fluorescence intensities (radiance) was carried out after removal of tissue autofluorescence by means of spectral unmixing as implemented by the IVIS image analysis software. Spectral unmixing allows for the discrimination of several distinct fluorescence spectra (i.e. IRDye 650 and tissue autofluorescence for this application) if their peaks differ by more than 10 nm.

Histology, autoradiography and ex vivo fluorescence microscopy

Excised tumors were embedded in optimal-cutting-temperature mounting medium (OCT, Sakura Finetek) and frozen on dry ice to cut a series of 10 μm frozen sections. Digital autoradiography was performed on a phosphor imaging plate (Fujifilm BAS-MS2325; Fuji Photo Film) at −20 °C. Phosphor imaging plates were read at a pixel resolution of 25 μm with a Typhoon 7000 IP plate reader (GE Healthcare). Then, the same frozen section was fixed in 4% paraformaldehyde for 12 minutes at room temperature. Whole-mount fluorescence images were acquired at ×40 magnification using a BX60 fluorescence microscope (Olympus America, Inc.)

equipped with a motorized stage (Prior Scientific Instruments Ltd.) and CC12 camera (Olympus). Whole-tumor montage images were obtained by acquiring multiple fields at ×40 magnification, followed by alignment using MicroSuite Biologic Suite (version 2.7, Olympus). Following fluorescence imaging, the same section was stained with H&E and whole-mount brightfield images acquired in a similar manner. Fluorescence and autoradiographic images were registered using Adobe Photoshop (CS6) as previously described (*1*).

1. Carlin S, Zhang H, Reese M, Ramos NN, Chen Q, Ricketts SA. A comparison of the imaging characteristics and microregional distribution of 4 hypoxia PET tracers. *Journal of nuclear medicine.* 2014;55(3):515-521.