Supporting Information

Markedly Enhanced Permeability and Retention Effects Induced by Photo-Immunotherapy of Tumors

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FULL METHODS

Cells. A431 (HER1 positive), 3T3/HER2 (HER2 positive), and MDA-MB-468 cells (HER1 positive) were used for PIT. Cells were grown in RPMI1640 supplemented with 10% FBS, 1% penicillin-streptomycin, and 0.03% L-glutamine in tissue culture flasks in a humidified incubator at 37˚C in an atmosphere of 95% air and 5% carbon dioxide.

Reagents. IRDye 700DX NHS ester (IR700; $C_{74}H_{96}N_{12}Na_4O_{27}S_6Si_3$, molecular weight of 1954.22) and IRDye 800CW NHS ester (IR800; $C_{50}H_{54}N_3Na_3O_{17}S_4$, molecular weight of 1166.20) were purchased from LI-COR Biosciences (Lincoln, NE). Texas Red-X, succinimidyl ester (TxRed) was obtained from Molecular Probes (Eugene, OR). Panitumumab, a fully humanized IgG2 monoclonal antibody (mAb) directed against the human epidermal growth factor receptor (EGFR; HER1) was purchased from Amgen (Thousand Oaks, CA). Trastuzumab, a recombinant humanized mAb directed against the human EGFR2 (HER2) was purchased from Genentech (South San Francisco, CA). PEGylated non-targeted quantum dot (Qtracker 800 non-targeted quantum dots; Qdot800) (Molecular Probes), SPIO (Feridex, Berlex Laboratories, Wayne, NJ), USPIO (SH U 555 C, Schering AG, Berlin, Germany), liposomal daunorubicin (DaunoXome; DX) (Galen US Inc., Souderton, PA) were used as nano-particles. Gd labeled G6-dendrimer was prepared as reported previously.[1] All other chemicals used were of reagent grade.

Synthesis of Dye-Conjugated MAbs. Conjugation of dyes with mAbs was performed according to the procedure reported previously.[2] Each mAb (1 mg, 6.8 nmol) was incubated with IR700 (60.2 μg, 30.8 nmol), IR800 (35.9 μg, 30.8 nmol), or TxRed (28.0 μg, 34.1 nmol) in 0.1 M Na_2HPO_4 (pH8.6) at room temperature for 1 h. The mixture was purified with a Sephadex G50 column (PD-10; GE Healthcare). The concentration of dye and protein was measured by absorption with spectroscopy (8453 Value System; Agilent Technologies) to confirm the number of fluorophore molecules conjugated to each mAb molecule. The number of IR700 and IR800 per antibody was adjusted to approximately four and two, respectively.

***In vivo* Nanodrug-Delivery After Photoimmunotherapy.** All *in vivo* procedures were conducted in compliance with the Guide for the Care and Use of Laboratory Animal Resources (1996), US National Research Council, and approved by the National Cancer Institute/NIH Animal Care and Use Committee. Six-week-old to 8-week-old female homozygote athymic nude mice were purchased from Charles River (National Cancer Institute, Frederick). During treatment, mice were anesthetized with isoflurane.

Two million A431 cells were injected subcutaneously in the right and left flanks of each mouse. Five days after cell injection, 100 μg of Pan-IR700 was administered intravenously, and 1 day later, one side was irradiated with NIR light from a red-light-emitting diode at wavelengths of 670-710 nm and a power density of 10-100 J/cm^2, as measured with an optical power meter (PM 100 (Thorlabs)). The other side was shielded from light using aluminum foil. One hour after PIT, Pan-IR800 (100 μg), Qdot800 (32.5

pmol), or liposomal daunorubicin (DaunoXome) (30 mg/kg) was injected intravenously, and *in vivo* dynamic imaging studies were carried out.

In vivo fluorescence images of IR700 and IR800 were obtained with a Pearl Imager (LI-COR Biosciences) using the 700 and 800 nm fluorescence channel. Qdot800 was detected with Maestro *in vivo* Imaging System (CRi Inc., Woburn, MA) using a band-pass filter, which ranges between 575 to 605 nm (excitation) and a long-pass NIR filter over 800 nm (emission). Fluorescence images of daunorubicin were also obtained with Maestro using a band-pass filter from 503 to 555 nm (excitation) and a long-pass green filter over 580 nm (emission). The tunable emission filter was automatically stepped in 10 nm increments from 650 to 950 nm and from 500 to 800 nm for the NIR and green filter sets at constant exposure. The spectral fluorescence images consist of autofluorescence spectra and the spectra from Qdot800 and daunorubicin, which were then unmixed, based on their spectral patterns using commercial software (Maestro software; CRi).

To validate that SUPR can be applied to a variety of tumor cell lines, five days after injection of two millions 3T3/HER2 cells in bilateral flanks, and seven days after MDA-MB-468 cells injection in bilateral mammary pads, tumor volumes of approximately 75 mm^3 were selected for the study. After PIT treatment using target-specific antibody conjugated with IR700 in each mouse model, Tra-IR800 and Pan-IR800 was administered into 3T3/HER2 mice and MDA-MB-468 mice, respectively, followed by dynamic imaging with Pearl Imager as mentioned above.

Dynamic contrast-enhanced (DCE) MR images were acquired with an Intera Achieva 3.0-T clinical scanner (Philips Medical Systems) using an in-house dedicated

mouse receiver coil. PAMAM dendrimer (generation 6th) loaded with Gd-DTPA (G6-Gd) was employed as a positive contrast material on T1 weighted image. SPIO (Superparamagnetic Iron Oxide) and USPIO (Ultrasmall Superparamagnetic Iron Oxide) were used as negative contrast agents on T2 weighted images. T1 and T2-weighted image sequences were applied in the coronal. G6-Gd were diluted to a final concentration of 6 mM Gd in PBS. A three dimensional (3D) T1W fast field echo imaging (T1-FFE) was performed (TR/TE = 16/23.3 msec, flip angle = 30˚, matrix = 512 × 256, FOV = 70 × 70 mm, coronal slices = 67, slice thickness = 0.17 mm, NSA = 6, scan time = ~4.5 min). SPIO and USPIO were diluted to a final concentration of 30 mg Fe/ml in PBS. For iron imaging, a fast field echo imaging (T2*-FFE) was employed (TR/TE = 277.7/9.23 msec, flip angle = 20˚, matrix = 512 × 256, FOV = 70 × 70 mm, coronal slice = 67, slice thickness = 0.20 mm, NSA = 6, scan time = ~5.5 min) with optimal TE time which tumors were seen as bright lesions before PIT. DCE MRI studies using G6-Gd, SPIO, and USPIO were performed separately with independent mice.

For administration of these agents, a 30-gauge needle was placed into the tail vein and extended using Tygon tubing (0.01 inch internal diameter). During the measurements, the breathing rates of the mice were monitored using a Biopac System MP150 (Biopac Inc.). Respiration rate was maintained at 25-30 respirations per min. Before intravenous injection of each agent, plain images were obtained with T1 or T2-weighted image sequences as previously described. After bolus injection of each agent at the amount of 100 μl, sequential DCE MR image series were acquired for ~30 min with the same sequence.

Mice were sacrificed with carbon dioxide immediately after *in vivo* imaging. The tumors were excised, and frozen or paraffin-embedded for histological study (H&E staining), fluorescence microscopy study, and Prussian blue staining after *ex vivo* imaging.

Biodistribution Study. In order to obtain the ultimate quantitative data, *in vivo* biodistribution study using ^{125}I labeled panitumumab (^{125}I-Pan) instead of Pan-IR800 was carried out. ^{125}I-Pan was prepared using the Iodo-Gen procedure, and the specific activity was 6.1 mCi/mg. One hour after PIT, ^{125}I-Pan (1 μCi/100 μg adjusted with intact panitumumab) was administered into mice. Whole-organ specimens were immediately removed and weighed 1 h postinjection, and the radioactivity was measured. The results were expressed as the percentage injected dose per gram (%ID/g).

Retention of Secondary Probes in PIT-Treated Tumors. To determine the retention of Qdot800 in the tumors after PIT, Qdot800 (32.5 pmol) was injected intravenously into A431 bearing mice at 1 h after PIT treatment, and the fluorescence images of Qdot800 were obtained at 1, 3, 6, and 24 h postinjection with Maestro *in vivo* Imaging System according to the protocol mentioned above.

Optimal Timing of Injection of Second Probes. To determine the optimal timing of second injection, Pan-IR800 (100 μg) was administered intravenously into A431 bearing mice at 1, 6, and 24 h after PIT treatment, and the dynamic imaging for 1 h was carried out with a Pearl Imager according to the protocol mentioned above.

Therapeutic Studies. To determine the effectiveness of PIT and DaunoXome, the following experiment was conducted: One million A431 cells were injected subcutaneously in the right flank of the mice. In order to determine the tumor volume, the greatest longitudinal diameter (length) and the greatest transverse diameter (width) were determined with an external caliper. Tumor volume based on caliper measurements was calculated using the following formula; tumor volume = length × width^2 × 0.5.[3] Tumors reaching approximately 40 mm^3 in volume were selected for the study. Selected mice were randomized into 4 groups of at least 10 mice per group for the following treatments: (1) no treatment; (2) liposomal daunorubicin (6 mg/kg); (3) PIT (50 J/cm^2); (4) PIT (50 J/cm^2) followed by liposomal daunorubicin (6 mg/kg) 1 h later. After treatment, the mice were monitored daily and their tumor volume was measured three times a week until it reached 750 mm^3, at which time mice were euthanized with carbon dioxide gas.

Fluorescence Microscopy. Ten-μm-thick frozen or paraffin sections were prepared and fluorescence was assessed using an Olympus BX51 microscope (Olympus America, Inc., Melville, NY) equipped with the following filters: excitation wavelength 590 to 650 nm, 360 to 370 nm, and 480 to 550 nm, emission wavelength 665 to 740 nm, 765 to 855 nm, and 590 nm long pass for IR700, Qdot800, and (daunorubicin, TxRed), respectively. Transmitted light differential interference contrast images were also acquired. H&E staining, platelet/endothelial cell adhesion molecule-1 (PECAM-1) immunohistochemical staining, Prussian blue staining were performed according to standard protocol.

Statistical Analysis. Data are expressed as means ± s.e.m. from a minimum of three experiments, unless otherwise indicated. Statistical analyses were carried out using a statistics program (GraphPad Prism; GraphPad Software). For multiple comparisons, a one-way analysis of variance (ANOVA) with post test (Kruskal-Wallis test with post-test) was used. The cumulative probability of survival, determining herein as the tumor volume was failed to reach 750 mm^3, were estimated in each group with the use of the Kaplan-Meier survival curve analysis, and the results were compared with use of the log-rank test with Bonferroni's correction for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Figure S1

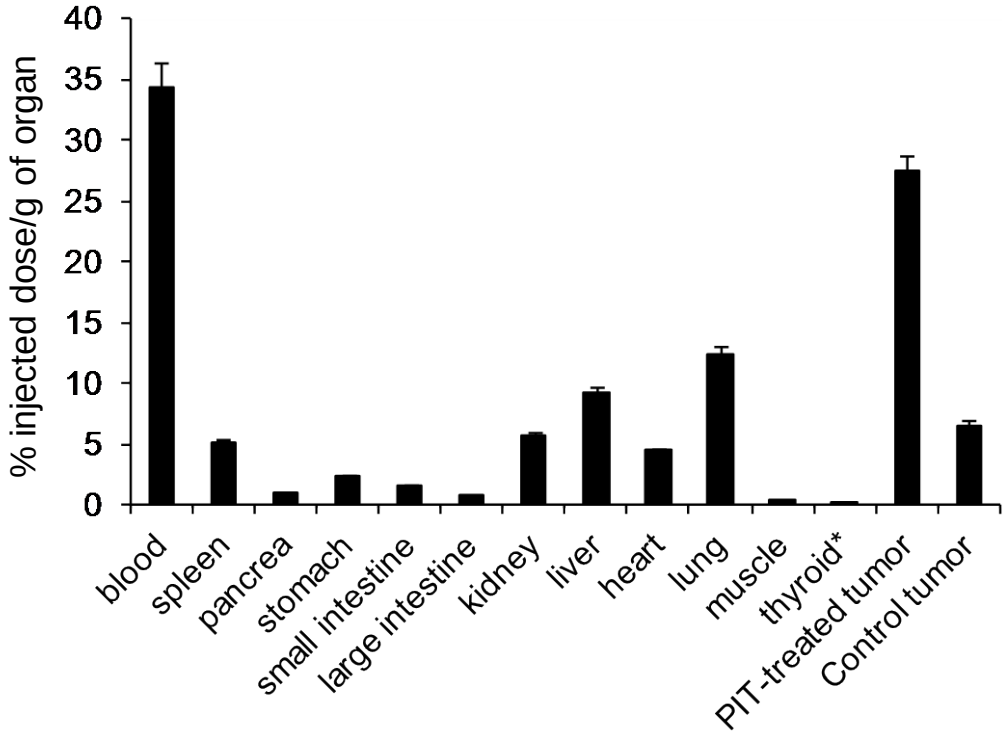

Figure S1. *In vivo* distribution of radioactivity at 1 h after injection of ^{125}I-labeled panitumumab into mice bearing A431 tumors treated by PIT. ^{125}I-labeled panitumumab was administered 1 h post-PIT. Expressed as % injected dose/g of organ. * % injected dose/organ. Data are means ± s.e.m. n = 4.

Figure S2

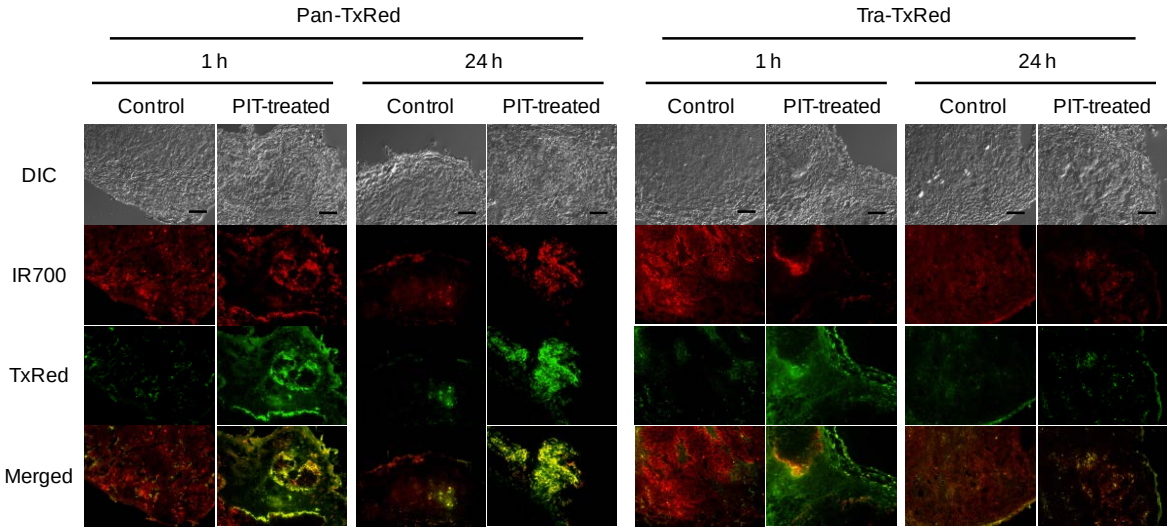

Figure S2. Target-specific binding of injected targeted antibody to surviving tumor cells in PIT treated tumors. Pan-TxRed or Tra-TxRed was administered to A431 bearing mice treated by PIT. One or 24 h later, fluorescence microscopic observation of frozen section excised tumors were performed. Pan-TxRed was rapidly and broadly distributed within PIT-treated tumors, and bound to surviving tumor cells efficiently up to 24 h postinjection of probe. DIC: differential interference contrast. IR700: red, TxRed: green. Scale bars, 200 μm.

Figure S3

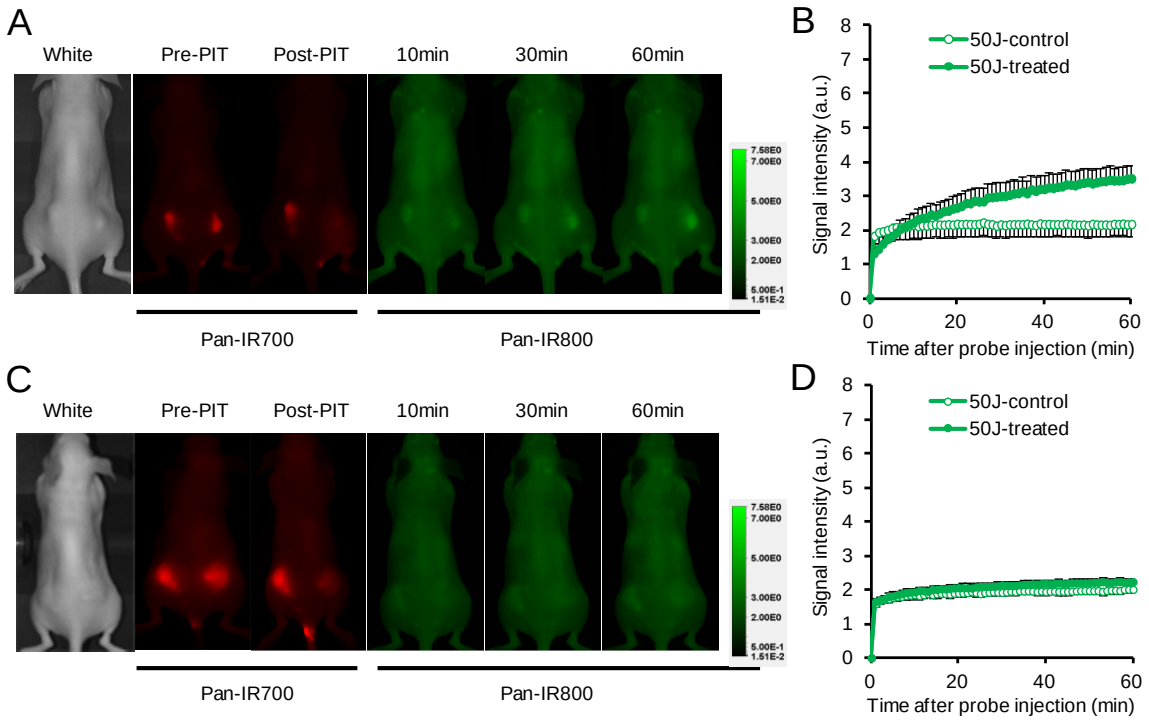

Figure S3. Nano-drug accumulation occurs maximally within the first 6 hours of Pan-IR700-mediated PIT. Only the right tumor received NIR light (50 J/cm^2) while the left tumor was covered. Dynamic fluorescence images of Pan-IR800 was obtained 6 (A and B) and 24 h (C and D) after PIT treatment. Data are means ± s.e.m. (n = 4) IR700: red, IR800: green.

Figure S4

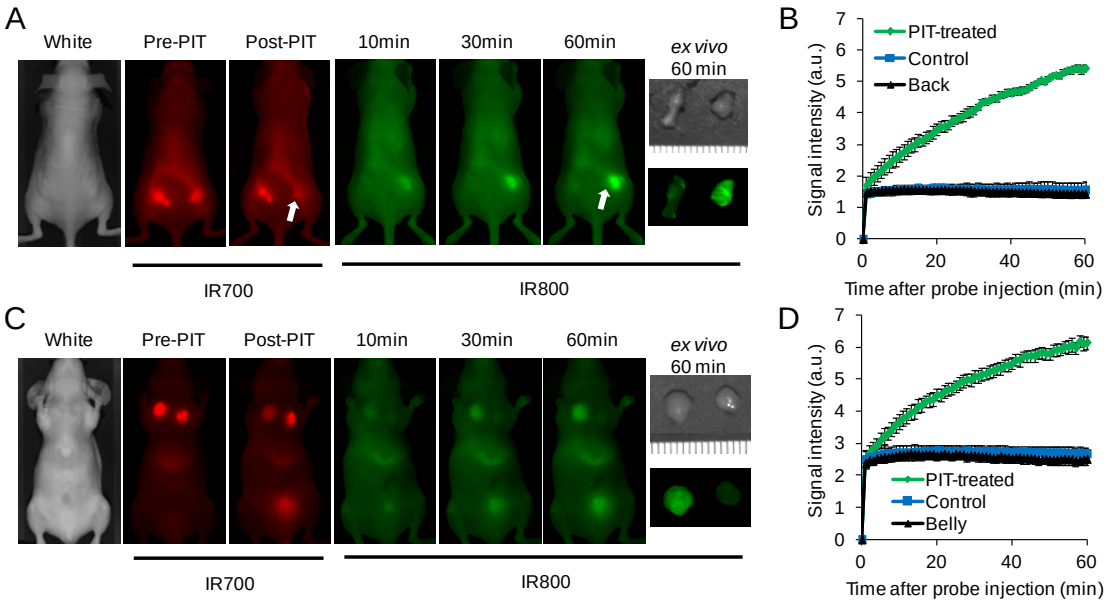

Figure S4. Feasibility of SUPR effect for various cancer cell lines (molecular targets). (A and B) Dynamic images of Tra-IR800 after Tra-IR700-mediated PIT. 3T3/HER2 tumor bearing mice were injected with Tra-IR700, and 24 h later, NIR light (50 J/cm^2) was applied to the right sided tumor while the left tumor was covered. Tra-IR800 was administered 1 h after PIT. White arrows show the sites where light was insufficiently irradiated to the 3T3/HER2 tumors. Tra-IR800 accumulated only in the regions where the tumor was exposed to NIR light. (C and D) Same experiments were performed with MDA-MB-468 tumor bearing mice (orthotopic model) injected with Pan-IR800 after Pan-IR700-mediated PIT. IR700: red, IR800: green.

Figure S5

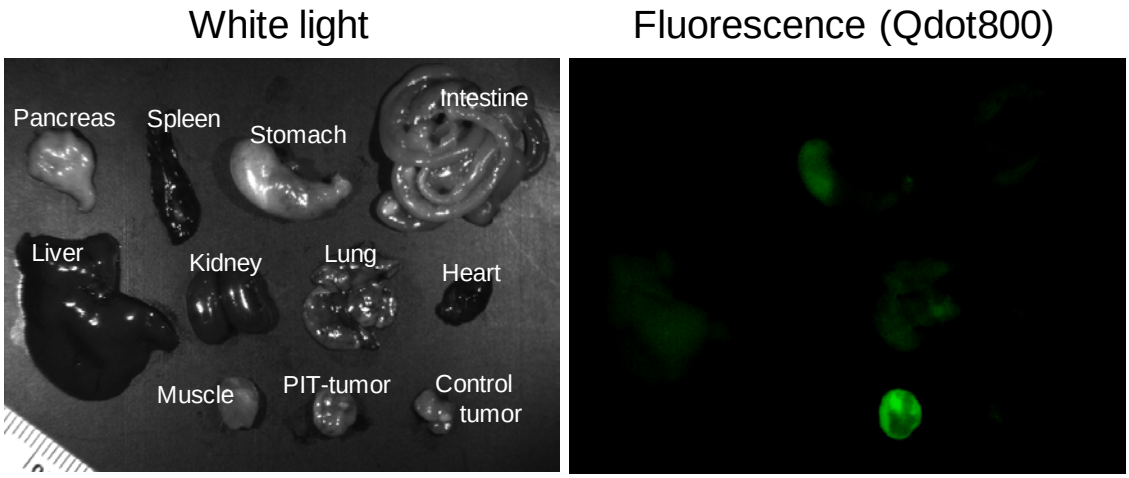

Figure S5. *Ex vivo* fluorescence imaging of Qdot800 in A431 bearing mice 1 h after PIT treatment. Highest uptake of Qdot800 was shown in PIT treated tumors.

Figure S6

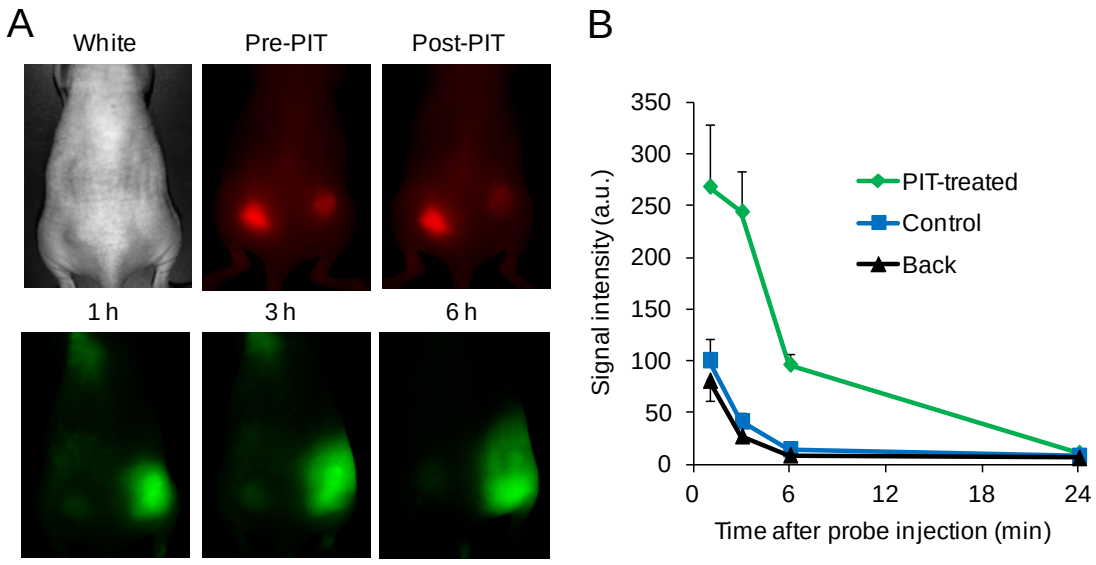

Figure S6. *In vivo* fluorescence imaging of Qdot800 in A431 bearing mice treated by PIT (only right tumor was exposed to light) over time. (A) High retention of Qdot800 in PIT treated tumors was maintained for 6 h.IR700: red, Qdot800: green. (B) Fluorescence signal intensity in PIT-treated tumors, control tumors, and back. Data are means ± s.e.m. (n = 3)

Figure S7

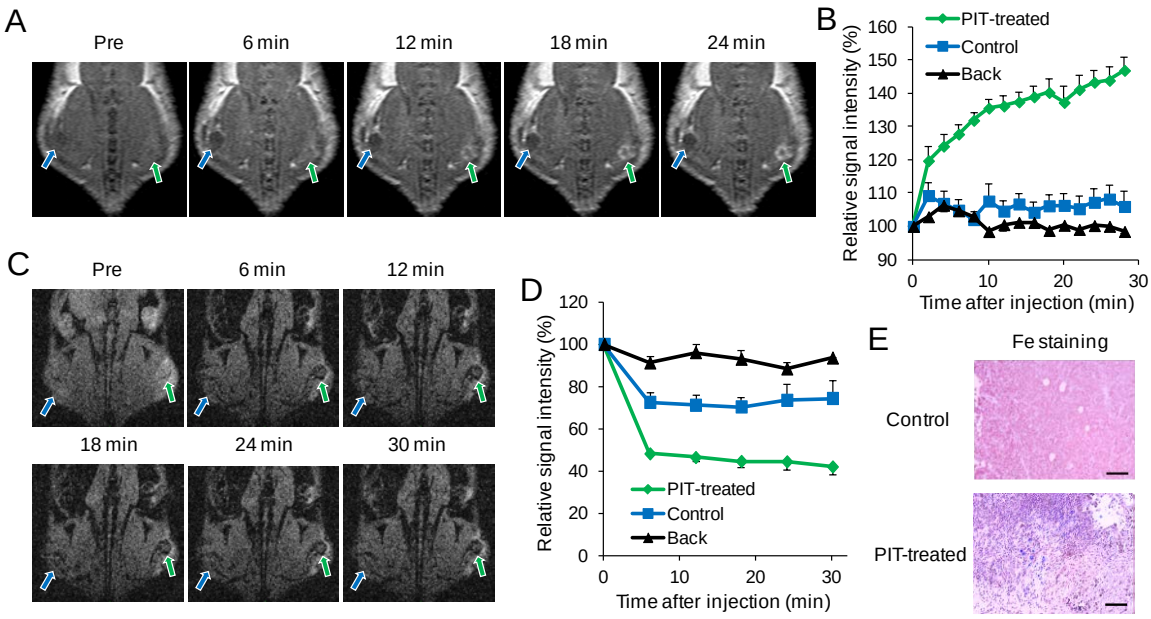

Figure S7. Enhanced delivery of nanoparticles can be documented with a variety of nanoparticulate MR contrast agents. (A) Dynamic T1-weighted images of G6-Gd (mean diameter = ~10 nm) after PIT. (B) Dynamic change of relative signal intensity in PIT-treated tumors, control tumors, and background. Data are means ± s.e.m. (n = 5) (C) Dynamic T2*-weighted images of USPIO (mean diameter = 20 nm) after PIT. (D) Dynamic change of relative signal intensity in PIT-treated tumors, control tumors, and background analyzed on MR images. Data are means ± s.e.m. (n = 4) (E) The uptake of USPIO in PIT-treated tumors was confirmed with Prussian blue staining. Scale bars, 200 μm.

Figure S8

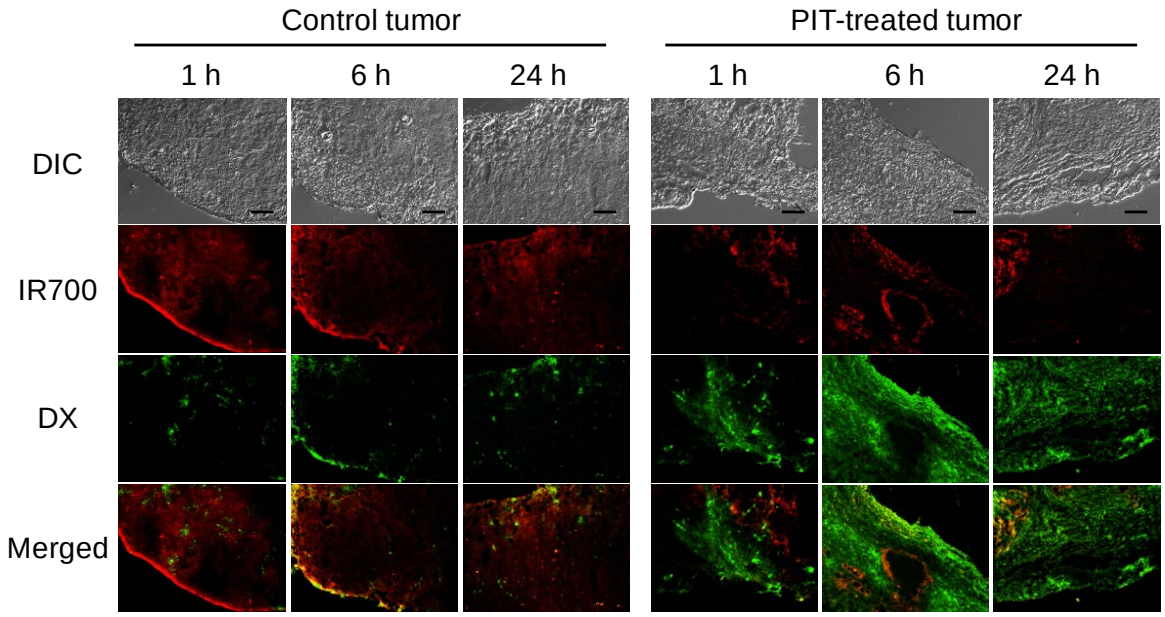

Figure S8. Liposomal daunorubicin distribute diffusely within the tumor and is retained a long time in PIT-treated tumors. Liposomal daunorubicin diffused throughout necrotic regions of PIT-treated tumors by 1 h postinjection, and was broadly distributed and retained by 6 h, colocalizing with surviving A431 tumor nests by 24 h. DIC: differential interference contrast, IR700: red, DX (DaunoXome): green. Scale bars, 200 μm.

Figure S9

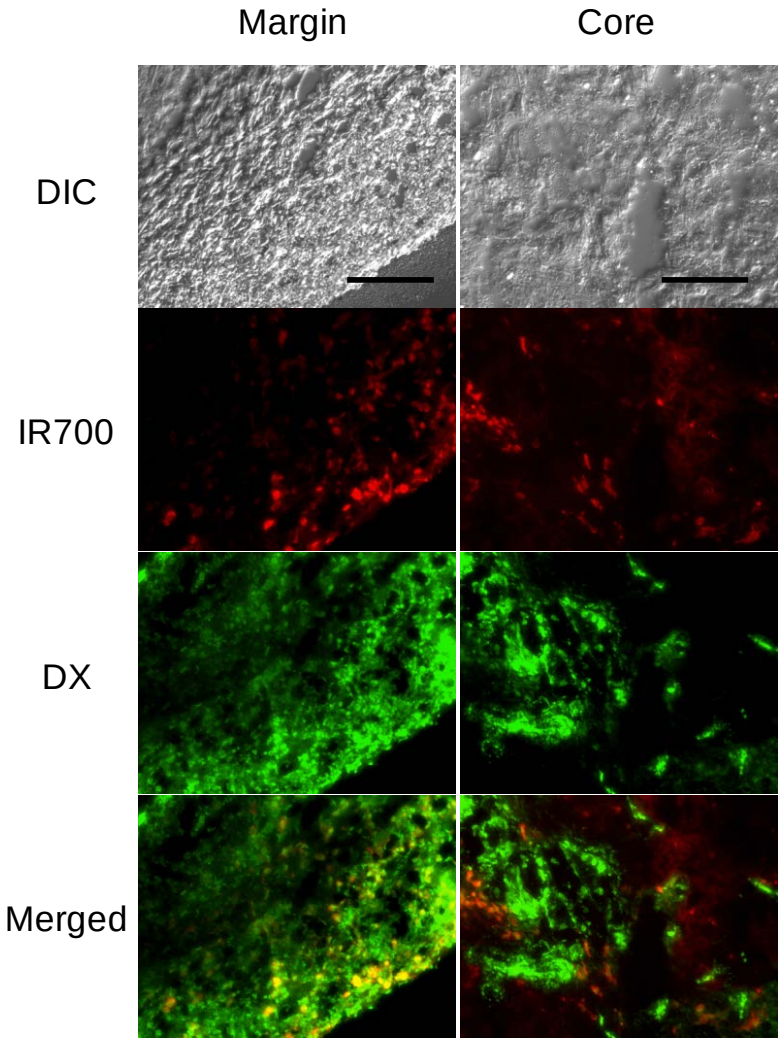

Figure S9. Liposomal daunorubicin was diffused both in the margin and core of the PIT-treated tumors 1 h after injection of probe. DIC: differential interference contrast, IR700: red, DX (DaunoXome): green. Scale bars, 200 μm.

Figure S10

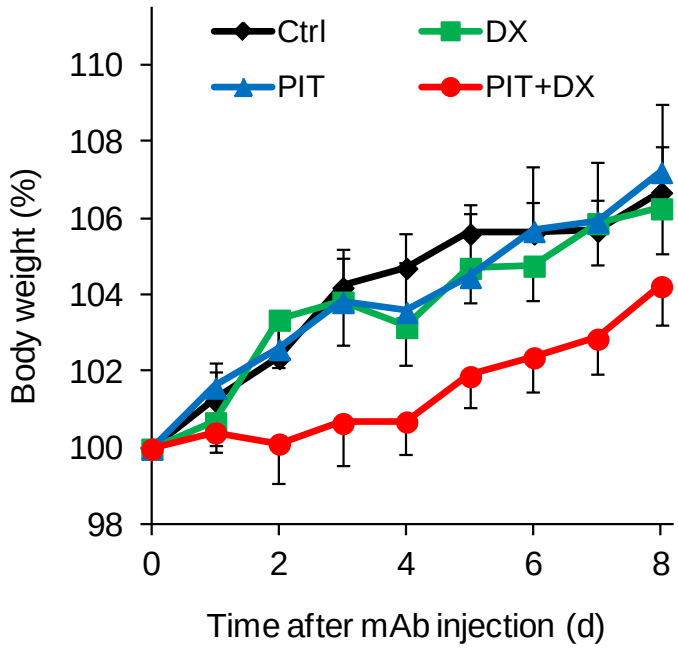

Figure S10. Body weight of tumor mice treated with DX only, PIT only, and DX 1 h after PIT in A431 bearing mice. The mice in the treatment study shown in Fig. 4 (E and F) were weighed during the study. The change of percent body weight is shown. Data are means ± s.e.m. ($n \geq 10$) DX: DaunoXome.

Supplementary Videos

Video S1. Dynamic fluorescence imaging of Pan-IR800 in A431 tumors. Only PIT-treated tumors were imaged within 1 h after injection of Pan-IR800.

Video S2. Dynamic fluorescence imaging of Qdot800 in A431 tumors. Only PIT-treated tumors were imaged within 1 h after injection of Qdot800.

Video S3. Dynamic magnetic resonance imaging of G6-Gd in A431 tumors. Only PIT-treated tumors were imaged within 1 h after injection of G6-Gd.

Video S4. Dynamic fluorescence imaging of liposomal daunorubicin in A431 tumors. Only PIT-treated tumors were imaged within 1 h after injection of liposomal daunorubicin.

REFERENCES AND NOTES

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