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Supporting Information

In vivo extracellular pH mapping of tumors using electron paramagnetic resonance

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Table of Contents

Cytotoxicity assay …………………………………………………………………… S-2

Animal preparation and tumor models …………………………………………………… S-2

Figure S1. 750 MHz EPR spectra of dR-SG at different pH values ………………………… S-4

Figure S2. Cytotoxicity test for the nitroxyl radicals R-SG ………………………………… S-5

Figure S3. Relation between EPR signal intensity and extracellular pH values …………… S-6

Figure S4. Time-course of EPR signal intensity of the R-SG probe ………………………… S-7

Figure S5. X-band EPR spectra of mouse urine ……………………………………………… S-8

References ………………………………………………………………………………… S-9

Cytotoxicity assay. The cytotoxicity assay was performed using HeLa cells according to a procedure described in the literature.[1] The R-SG probe was tested together with three other nitroxyl radicals which are commonly used in EPR studies: 3-Carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl (3-carboxy-PROXYL), 3-Carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (3-carbamoyl-PROXYL), and 4-Oxo-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempone). All chemicals were analytical grade and purchased from Wako Pure Chemical Industries (Osaka, Japan), Tokyo Chemical Industries (Tokyo, Japan) and Sigma-Aldrich (Tokyo, Japan), respectively.

HeLa cells were seeded at 1×10^3 cells/well in a 96-well microplate (BioLite 96 well multidish, Thermo Fisher Scientific, Waltham, MA, USA), in Dulbecco's modified Eagle medium (DMEM, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum and 3.7 g/L $NaHCO_3$ at 37 °C in 5% CO_2 / 95% air. After 24 hours, the medium was replaced with DMEM containing 0, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 0.1, 1.0, 10, and 100 mM of the nitroxyl radicals. The cells were incubated for an additional four days. After the incubation period, the cells were fixed by methanol for 10 min, and stained with 0.1% crystal violet in methanol/water (20:80; v/v) for 1 hour. Methanol, 200 mL/well, was added to stained cells, and the microplate was shaken for 20 min. Cell viability was quantified by measuring the absorbance at 570 nm with a microplate reader (model 680, Bio-Rad, Hercules, CA).

Animal preparation and tumor models. Six-week-old C3H/HeJ male mice and six-week-old BALB/c-nu/nu male mice were purchased from Japan SLC (Hamamatsu, Japan). Murine squamous cell carcinoma (SCC VII) cells[2] were used as a tumor model for the C3H/HeJ mice. The SCC VII cells were cultured as previously reported.[3] An SCC VII cell line – tested in 2015 by Chromosome Science Labo Inc. (Sapporo, Japan) using the FISH test for identification of animal species – was kindly obtained from Dr. Shin-ichiro Masunaga (Kyoto University, Kyoto, Japan). Approximately a million SCC VII cells were subcutaneously injected into the right hind legs of the mice and in vivo pH mapping was performed 5 and 8 days after the implantation.

Human-derived pancreatic ductal adenocarcinoma cell lines, MIA PaCa-2, SU.86.86, and Hs766t, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Hs766t cells and MIA PaCa-2 cells were maintained in Dulbecco's modified Eagle medium (Gibco-BRL/Thermo Fisher Scientific Co., Carlsbad, CA, USA) supplemented with 10% fetal

bovine serum (FBS) at 37°C in 5% CO_2. SU.86.86 cells were maintained in RPMI 1640 medium (Gibco-BRL/Thermo Fisher Scientific Co., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS). Approximately ten million cells of MIA PaCa-2, SU.86.86, or Hs766t were subcutaneously injected into the right hind legs of the BALB/c-nu/nu mice. Prior to in vivo pH mapping, tumors were developed until their longitudinal length reached 12 mm. Time required for tumor development: MIA PaCa-2, 2–3 weeks; SU.86.86, 4–6 weeks; Hs766t, 4 weeks.

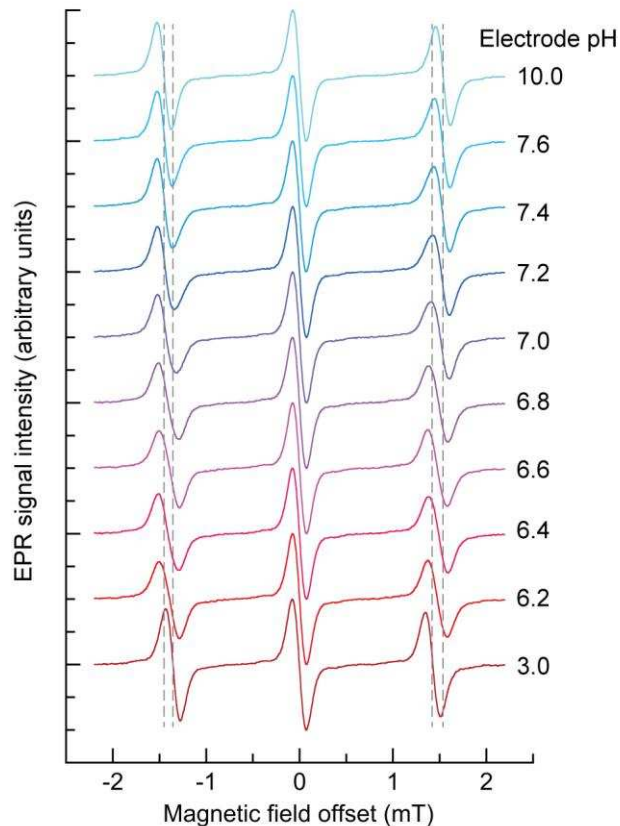

Figure S1. 750 MHz EPR spectra of 2 mM dR-SG (pKa = 6.6 at 37 °C) in PBS at different pH values. The solution pH was adjusted by adding HCl or NaOH. EPR spectra were measured at room temperature. EPR spectrometer settings were: scan time 0.1 s, scanning magnetic field 9.0 mT, magnetic field modulation 0.15 mT, modulation frequency 90 kHz, lock-in amplifier time-constant 30 μs, number of data points 2048 per scan, and incident RF power 2.2 mW.

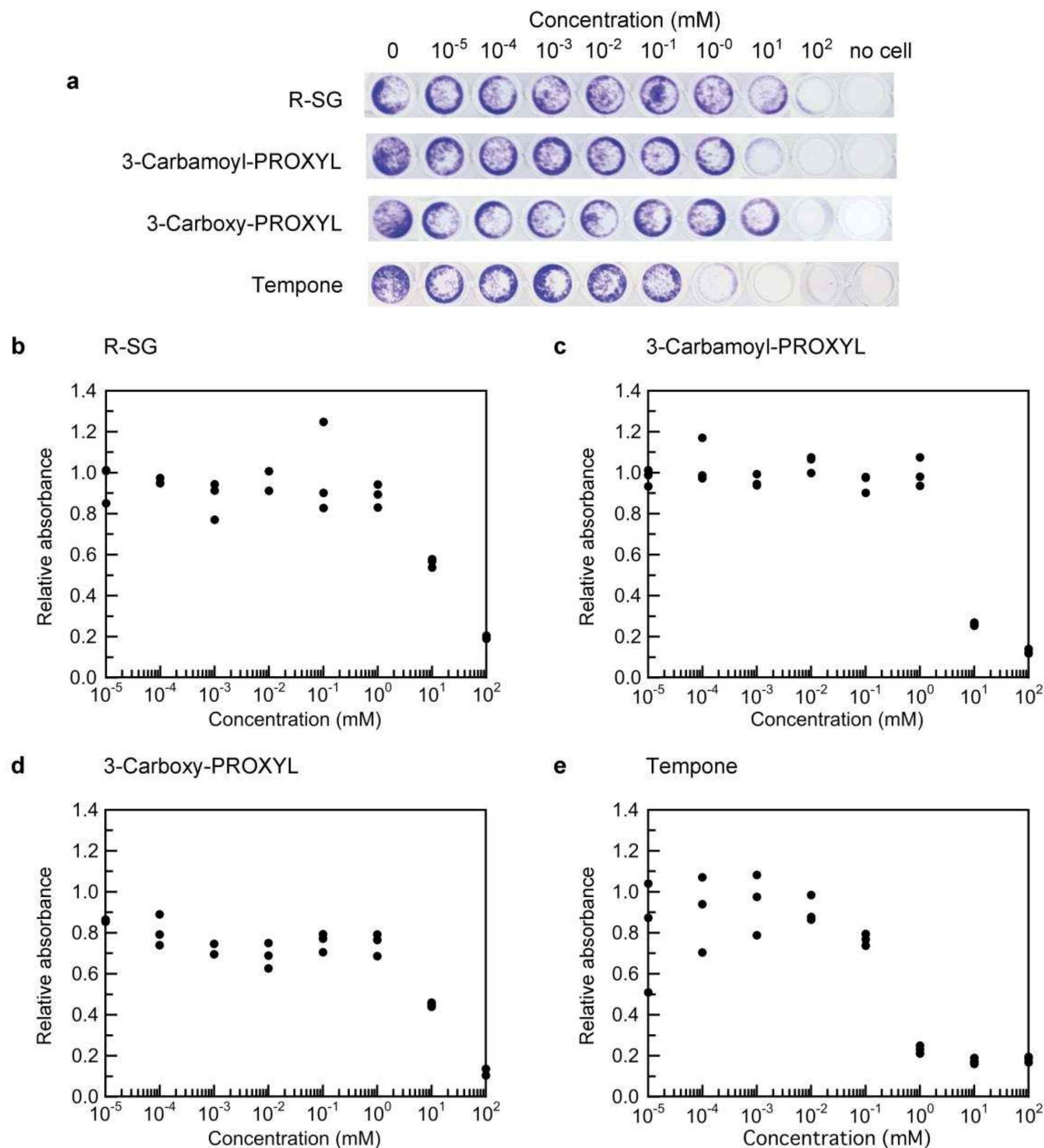

Figure S2. Cytotoxicity test for the nitroxyl radicals R-SG, 3-carbamoyl-PROXYL, 3-carboxy-PROXYL and Tempone. (a) Photograph of the microplate with stained HeLa cells which were incubated in the presence of different concentrations of the nitroxyl radicals for four days. (b–e) Survival fraction of the cells (relative absorbance at 570 nm) for different concentrations of R-SG, 3-carbamoyl-PROXYL, 3-carboxy-PROXYL and Tempone, respectively. Data points represent the data from three independent experiments.

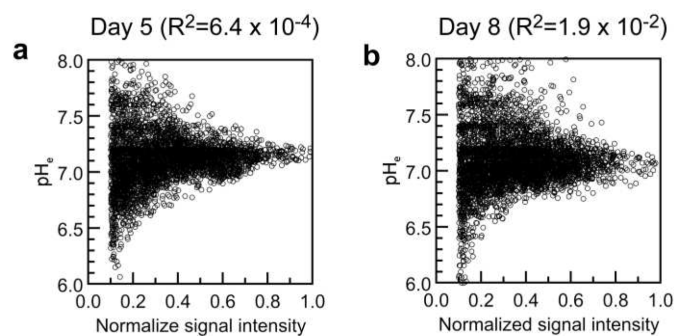

Figure S3. Relation between EPR signal intensity and extracellular pH values. The scatter plots for EPR signal intensity and measured pH_e for SCC VII tumor-bearing mice at (a) day 5 and (b) day 8 were generated from the data used in Fig. 3 of the main text. Only 20% of the total data is shown for clarity, because the number of datapoints in the full dataset is too large; 14,504 points are plotted for the day 5 plot and 16,361 for the day 8 plot. The data with signal intensity greater than 10% of the maximum are plotted. The coefficient of determination R^2 was below 0.02 in both plots.

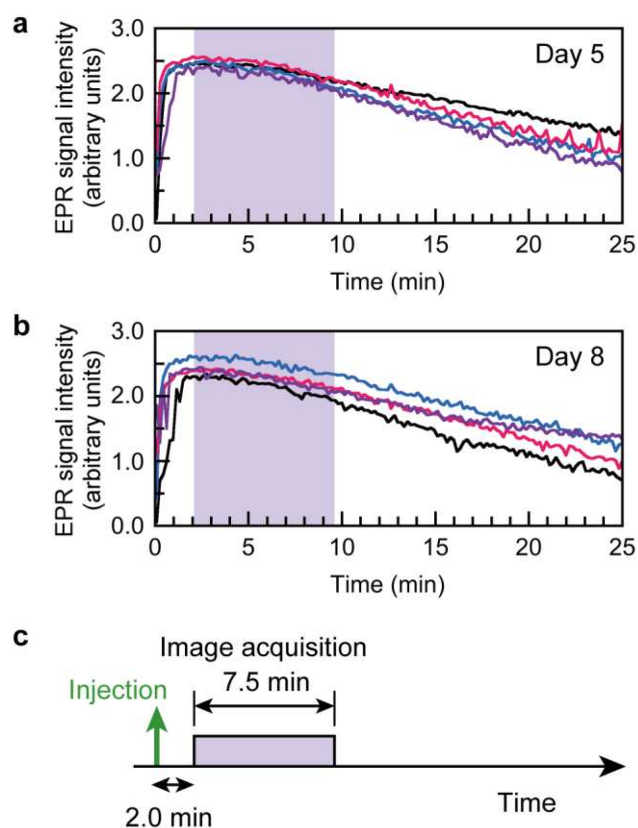

Figure S4. Time-course of EPR signal intensity of the R-SG probe measured from SCC VII tumor-bearing mouse legs on day 5 (a) and day 8 (b) after tumor cell implantation. The R-SG radical was administered to the subject mice through the tail vein catheter as a bolus (150 µl of 67 mM solution, corresponding to 6.7 mg or 0.4 mmol/kg body weight). The R-SG solution was prepared in pure water, and the pH was adjusted to 7.4 by addition of NaOH. The solid lines show the EPR signal intensity for 4 individual measurements. EPR spectrometer settings were as follows: scan time 0.1 s, scanning magnetic field 9.0 mT, magnetic field modulation 0.2 mT, modulation frequency 90 kHz, lock-in amplifier time-constant 100 µs, number of data points 2048 per scan, and incident RF power 2.2 mW. (c) Time chart of data acquisition for EPR imaging experiments. EPR projections were recorded within a 7.5 min period starting from 2 min after injection of the bolus of the probe.

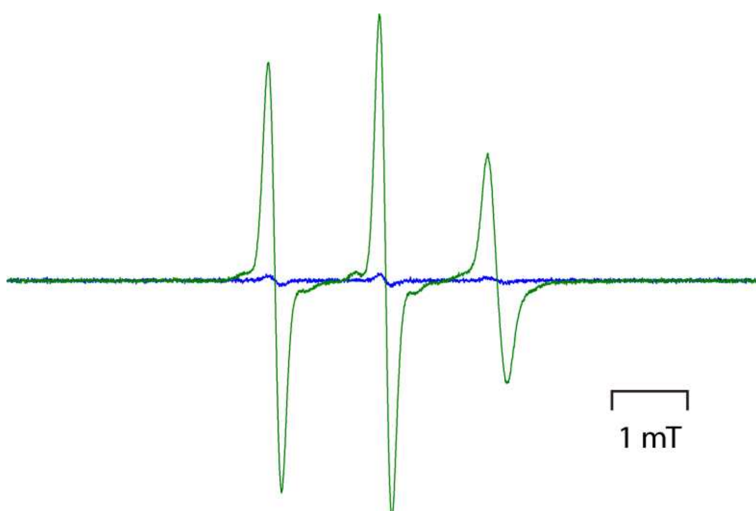

Figure S5. X-band EPR spectra of mouse urine collected 30 – 60 min after intravenous injection of the R-SG radical (150 µl, 67 mM). (Blue) EPR spectrum of mouse urine diluted 2 times with PBS. (Green) The same as (blue) but in the presence of 5 mM $K_3[Fe(CN)_6]$. The urine of three different animals was measured, yielding similar results. EPR spectra were recorded with a Bruker EMX spectrometer (Billerica, MA, US) using the following settings: scan time 20 s, time constant 5.12 ms, magnetic field sweep 10.0 mT, modulation amplitude 0.2 mT, modulation frequency 100 kHz, microwave power 20 mW. After the addition of $K_3[Fe(CN)_6]$, a strong EPR signal from dR-SG appeared. Thus, a significant amount of the radical exists in the reduced form as the corresponding hydroxylamine in the urine.

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