Supplemental Methods

Cells

AsPC-1, Sk-Br-3, U-87 MG, BxPC-3, and MIA PaCa-2 were obtained from ATCC (Rockville, MD). AsPC-1 and BxPC-3 cells were cultivated in RPMI 1640 medium (Wako Pure Chemical Industries, Osaka, Japan), and Sk-Br-3, U-87 MG and MIA PaCa-2 cells were cultivated in D-MEM (High Glucose) medium (Wako Pure Chemical Industries). All media were supplemented with 10% fetal calf serum (Nichirei Biosciences, Tokyo, Japan). All cell lines were cultivated in humidified atmosphere containing 5% carbon dioxide at 37°C.

Animals

BALB/c-nu/nu male mice were obtained from Japan SLC (Shizuoka, Japan). Male mice were injected subcutaneously with 3×10^6 AsPC-1, 2×10^6 BxPC-3, 1×10^7 MIA PaCa-2, or 5×10^6 U-87 MG cells. BxPC-3 and MIA PaCa-2 cells were co-injected with BD Matrigel Matrix (BD Biosciences, Bedford, MA).

Cellular uptake studies

Cells were seeded into 24-well plates (1×10^5 cells in 500 μL per well) 18 h before experiments and then incubated at 37°C to form nearly confluent monolayers. The cells were rinsed three times with PBS, and incubated with approximately 15 MBq of [^{11}C]MALA or

18.50 kBq of [^{3}H]ALA in 500 μL of PBS with 0.1% glucose at 37°C. After 10 min incubation, the cells were washed three times with ice-cold PBS. The cells were dissolved in 0.1 M NaOH aqueous solution (100 μL), and radioactivity of ^{11}C was measured with an auto-well γ counter. Radioactivity of ^{3}H was measured using a liquid scintillation counter after addition of scintillation cocktail (Hionic-Fluor, PerkinElmer, Waltham, MA, 1 mL). The total protein concentration in samples was determined by Bradford protein assay (Bio-Rad, Richmond, CA).

Western blotting

Cells were lysed in lysis buffer (Cell Signaling Technology, Danvers, MA) containing 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (Sigma, St. Louis, MO). Protein concentrations were measured by Bradford protein assay. The cell lysates (40 μg) were heated at 95°C for 5 min in SDS sample buffer (50 mM Tris-HCl pH 6.8, 12.5% glycerol, 1% sodium dodecylsulfate, 0.01% bromophenol blue) containing 5% 2-mercaptoethanol, separated by SDS-PAGE, and then transferred to a polyvinylidene fluoride (PVDF) membrane (Hybond-P, GE Healthcare, Little Chalfont, UK) using a Trans-Blot Semi-Dry Transfer cell (Bio-Rad). The membrane was blocked with 3% (w/v) perfect-block (Mo Bi Tec, Gottingen, Germany) in TBS with 0.2% Tween-20, and then

incubated with anti-ALAD (1:100, Atlas Antibodies AB, Stockholm, Sweden) or anti-β-actin antibodies (1:20000, Sigma). Primary antibodies were detected using HRP-conjugated goat anti-rabbit or anti-mouse IgG (GE Healthcare) and visualized using an ECL Prime kit (GE Healthcare).

In vitro accumulation of ALA-induced PpIX

Cells were seeded into 6-well plates (3×10^5 cells in 2 mL per well) 18 h before experiments and then incubated at 37°C to form nearly confluent monolayers. The cells were rinsed three times with PBS, and incubated in serum free medium with or without 1 mM ALA (Wako Pure Chemical Industries) at 37°C. After 4 h incubation, the cells were washed three times with ice-cold PBS, and brought into 60 μL of a solvent containing 1 M aqueous perchloric acid and methanol 1:1 (v/v) by scraping with a cell scraper (Costar, Cambridge, MA). After centrifugation at 10,000 × g for 1 min to remove the cell debris, fluorescence of each supernatant (50 μL) was measured with a spectrophotometer. Samples were excited at 405 nm, and fluorescence emission was scanned at 620 nm. The amount of PpIX was determined by measurement of standard PpIX (Sigma) solution.

Biodistribution studies in normal or tumor-bearing mice

Biodistribution studies were performed when xenograft tumors reached a diameter of

about 10 mm. Normal or tumor-bearing mice were intravenously injected with the mixture of [^{11}C]MALA (16.00 MBq for normal mice or 9.15 ± 2.69 MBq for tumor-bearing mice) and [^{3}H]ALA (18.50 kBq). After sacrificing mice, the blood and organs of interest were removed and weighed. Radioactivity of ^{11}C was measured with auto-well γ counters. After the radioactivity of ^{11}C decayed to the background level, organs (50 to 200 mg) were solubilized in 1 mL of tissue solubilizer (Soluen-350, PerkinElmer) at 60°C. Blood (50 μL) was decolorized with aqueous dihydrogen dioxide solution (100 μL), followed by solubilization with Soluen-350. Hionic-Fluor (9 mL) was added to the solution of organs and blood, for each. ^{3}H radioactivity was measured using a liquid scintillation counter.

In vivo stability of [^{11}C]MALA

[^{11}C]MALA (50.06 MBq) was intravenously injected into mice bearing AsPC-1 xenograft tumors and sacrificed at 10 min post-injection. Plasma (200 μL) was isolated from whole blood by centrifugation at 15,000 rpm for 4 min at 4°C, deproteinized by addition of acetonitrile (600 μL), and then centrifuged at 15,000 rpm for 4 min at 4°C. The organs of interest were removed, homogenized in acetonitrile and centrifuged at 15,000 rpm for 4 min at 4°C. Layers were separated and counted with an auto-well γ counter. Radioactivity in supernatants was analyzed by radio high-performance liquid chromatography (HPLC) on a

hydrophilic interaction chromatography column (COSMOSIL HILIC 4.6 mm ID × 150 mm, Nacalai Tesque, Kyoto, Japan) with the mobile phase of 30 mM aqueous ammonium acetate and acetonitrile (25:75 v/v) at the flow rate of 1.0 mL/min.

Dynamic PET imaging

Dynamic PET scans were performed for 90 min (5 × 1 min, 5 × 5 min, 3 × 10 min, and 2 × 15 min) using a small-animal PET system (Inveon, Siemens Medical Solutions, Malvern, PA) immediately after [^{11}C]MALA injection (11.35 ± 0.94 MBq). Body temperature was maintained at approximately 37°C by lamp and heating pad during scans. Images were reconstructed using a 3D maximum *a posteriori* (18 iterations with 16 subsets, β = 0.2) without attenuation correction.

***In vivo* tumor accumulation of PpIX induced by exogenous ALA**

At 4 h post-injection of ALA (6 mg), tumor-bearing mice were sacrificed and tumors were removed. Approximately 0.05 to 0.2 g of tumors were homogenized in 1 mL of a solvent containing 1 N aqueous perchloric acid and methanol 1:1 (v/v) using Mixer Mill MM300 (QIAGEN KK, Tokyo, Japan). The homogenized mixtures were centrifuged at 1,000 × g for 10 min at 4°C, and fluorescence of each supernatant (200 μL) was measured as described in the *in vitro* studies (n = 4).

Ex vivo autoradiography, immunofluorescence, and florescence microscopy

At 60 min post-injection of [^{11}C]MALA (84.91 MBq) (4 h post-injection of ALA), mice were sacrificed and AsPC-1 tumors were removed and frozen in optimal-cutting-temperature compound (Sakura, Tokyo, Japan). Dried sections (20 μm thick) were exposed to an imaging plate (Fuji Film, Tokyo, Japan) for 20 min to determine the radioactivity of [^{11}C]MALA. After autoradiographic exposure, sections were fixed with 4% paraformaldehyde, blocked with 3% (w/v) bovine serum albumin in PBS with 0.2% Tween-20, and then incubated with the anti-ALAD antibody (1:100). Primary antibody was detected using Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Invitrogen, Carlsbad, CA). Alexa Fluor 488 and PpIX signals were observed using a fluorescence microscope (BX53, Olympus, Tokyo, Japan).

Supplemental Table 1. *In vivo* biodistribution of [^{11}C]MALA in normal mice after intravenous injection.

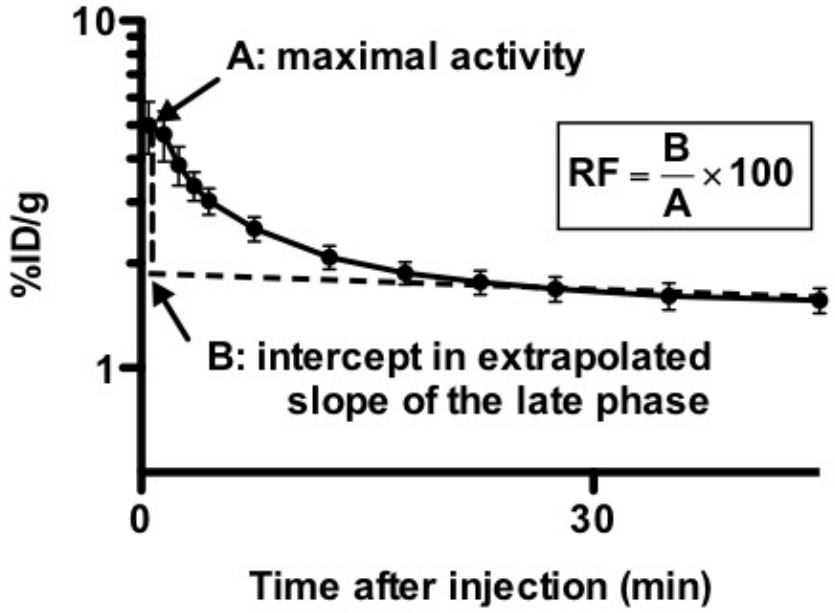
	1 min	10 min	30 min	60 min	90 min
Blood	10.54 ± 0.93	3.94 ± 1.80	1.78 ± 0.16	1.56 ± 0.45	1.00 ± 0.15
Brain	0.37 ± 0.03	0.20 ± 0.06	0.15 ± 0.02	0.14 ± 0.01	0.10 ± 0.01
Heart	5.07 ± 0.53	2.14 ± 0.31	1.62 ± 0.13	1.41 ± 0.07	0.95 ± 0.06
Lung	9.52 ± 0.81	4.31 ± 0.59	3.03 ± 0.53	2.14 ± 0.27	1.33 ± 0.25
Liver	8.92 ± 0.75	10.75 ± 0.58	5.30 ± 0.67	3.71 ± 0.54	2.23 ± 0.31
Spleen	6.03 ± 0.79	15.20 ± 0.60	10.10 ± 1.32	6.79 ± 0.93	5.08 ± 1.88
Pancreas	3.14 ± 0.21	2.26 ± 0.09	1.96 ± 0.52	1.61 ± 0.19	1.12 ± 0.17
Stomach	5.03 ± 0.34	3.12 ± 0.33	1.86 ± 0.20	1.78 ± 0.31	1.05 ± 0.08
Intestine	7.47 ± 1.06	7.34 ± 0.57	4.90 ± 0.73	4.20 ± 0.88	2.48 ± 0.28
Kidney	61.63 ± 7.95	91.30 ± 6.53	51.32 ± 8.20	32.78 ± 4.44	19.30 ± 2.66
Muscle	2.64 ± 0.13	1.04 ± 0.12	0.83 ± 0.30	0.61 ± 0.09	0.80 ± 0.95
Bone	4.60 ± 0.84	3.06 ± 0.12	1.77 ± 0.51	1.50 ± 0.44	1.09 ± 0.49

Data represent the mean of %ID/g ± SD.

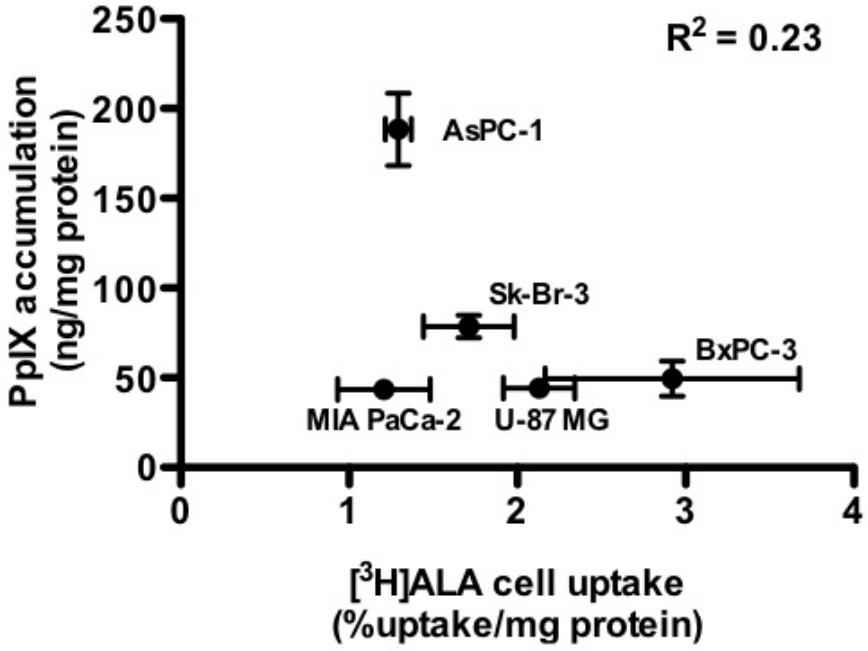
Supplemental Table 2. *In vivo* biodistribution of [^{3}H]ALA in normal mice after intravenous injection.

	1 min	10 min	30 min	60 min	90 min
Blood	7.01 ± 0.85	2.12 ± 0.39	1.57 ± 0.15	1.14 ± 0.21	1.16 ± 0.13
Brain	0.75 ± 0.14	0.64 ± 0.11	0.99 ± 0.10	0.97 ± 0.15	0.59 ± 0.28
Heart	3.63 ± 0.36	1.22 ± 0.16	0.98 ± 0.09	0.83 ± 0.18	0.43 ± 0.06
Lung	5.93 ± 0.50	2.58 ± 0.25	1.70 ± 0.28	1.31 ± 0.16	1.02 ± 0.18
Liver	7.28 ± 0.60	11.85 ± 1.07	11.03 ± 0.76	12.59 ± 0.63	10.36 ± 0.42
Spleen	2.70 ± 0.67	2.60 ± 0.65	1.88 ± 0.52	1.24 ± 0.17	0.82 ± 0.15
Pancreas	3.54 ± 0.42	2.15 ± 0.08	1.91 ± 0.26	1.72 ± 0.17	1.18 ± 0.26
Stomach	2.77 ± 0.13	2.43 ± 0.15	1.94 ± 0.44	1.65 ± 0.27	1.17 ± 0.44
Intestine	4.41 ± 0.31	2.50 ± 0.20	2.17 ± 0.21	1.70 ± 0.24	1.18 ± 0.11
Kidney	23.46 ± 2.04	14.48 ± 1.94	9.52 ± 0.24	7.15 ± 0.78	5.25 ± 1.00
Muscle	2.59 ± 0.35	1.64 ± 0.28	1.18 ± 0.14	1.04 ± 0.12	0.76 ± 0.28

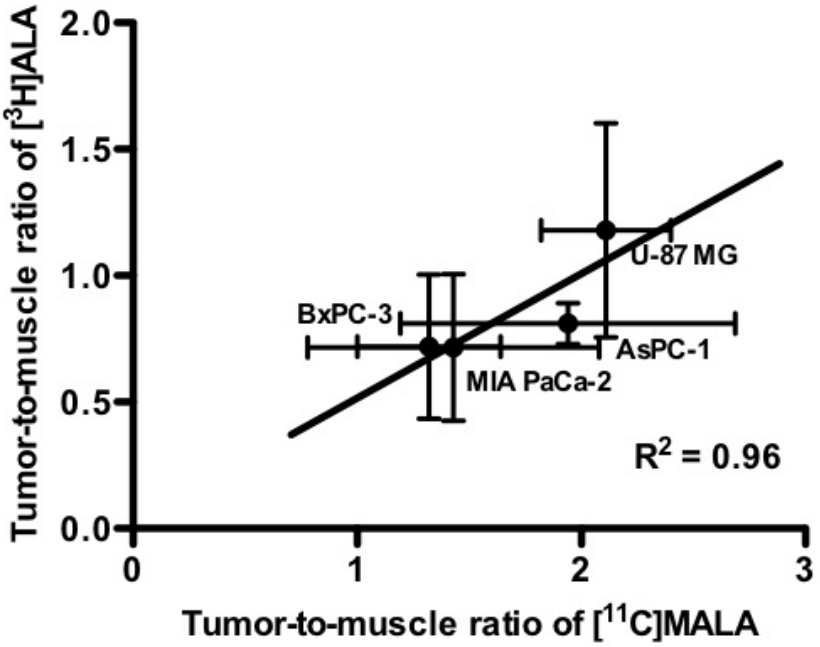
Data represent the mean of %ID/g ± SD.



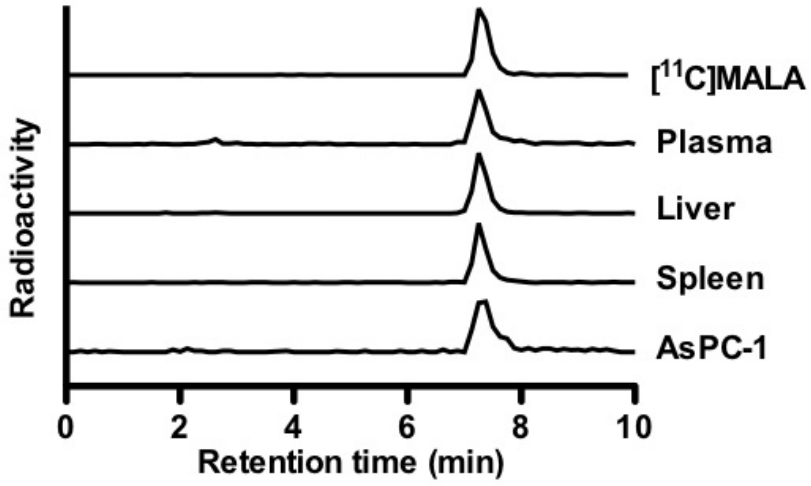
Supplemental Figure 1. Analysis of time-activity curves (TACs). Retention fraction (RF) was determined by dividing the intercept in extrapolated slope of the late phase of TAC back to the time of maximal activity (B) by maximal activity (A).



Supplemental Figure 2. The correlation between cellular radioactivity of [^{3}H]ALA in the cell uptake studies and ALA-induced PpIX accumulation. Error bars indicate SD (n = 3).



Supplemental Figure 3. The correlation between *in vivo* tumor-to-muscle ratios of [^{11}C]MALA and [^{3}H]ALA at 1 min post-injection. Error bars indicate SD (n = 4).



Supplemental Figure 4. *In vivo* stability of [^{11}C]MALA. HPLC profiles of radioactivity of [^{11}C]MALA (injected sample); plasma; and homogenates of liver, spleen and AsPC-1 tumor after deproteinization.