SUPPLEMENTAL MATERIALS

MATERIALS AND METHODS

Reagents and Cell Culture

$^{64}CuCl_2$ was purchased from the Department of Medical Physics, University of Wisconsin at Madison (Madison, WI). The pH was adjusted to 7.0, and the $^{64}CuCl_2$ solution was diluted with phosphate-buffered saline (PBS). ^{64}Cu-DOTA was prepared based on the previous procedure (34). The pH was adjusted to 7.0, and the $^{64}CuCl_2$ solution was diluted with phosphate-buffered saline (PBS). B16F10 murine melanoma cells and A375M human melanoma cells were cultured in Dulbecco's modified Eagle's high-glucose medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). All other cells were cultured using the ATCC recommended medium. The cells were maintained at 37°C in a humidified 95% air and 5% CO_2 incubator.

In Vitro studies

Western Blot Analysis of CTR1 Expression

CTR1 expression in tumor cells and tissues was detected by a Western blot assay. The cells (H460, 22B, SKOV-3, CT26, A375M, 4T1, U87MG, HT-29, 67NR, MDA-MB-435, B16F10 and PC-3) were washed three times with PBS and lysed in T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific Inc., Rockford, IL, USA). Tumor tissue lysate (B16F10 and A375M) was prepared by homogenizing the tumor specimens in the T-PER Tissue Protein Extraction Reagent. The supernatant was collected by centrifugation at 14,000 rpm for 15 min at 4°C. Protein concentrations were measured using the Bradford assay (BioRad). A total of 100 μg of total protein from each sample was separated on a 4-12% NuPAGE Bis-Tris gel by

electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane (Invitrogen Corp., Carlsbad, CA, USA). After blocking with tris-buffered saline containing 5% nonfat milk for 1 h, the membrane was incubated overnight at 4°C with rabbit polyclonal anti-CTR1 primary antibody (Santa Cruz biotechnology, Inc. Santa Cruz, CA) (1:1000) and rabbit polyclonal antibody to β-actin (Abcam Inc. Cambridge, MA) (1:1000), followed by a 1 h incubation at room temperature with goat polyclonal secondary antibody to rabbit IgG – H&L (HRP) (Abcam Inc. Cambridge, MA) (1:5,000). After extensive washing, the protein bands were visualized using enhanced chemiluminescence (ECL) Western blotting substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA). To determine the relative CTR1 protein levels, the intensity of the CTR1 protein band was normalized with the intensity of the β-actin protein band from each sample.

In Vitro Cell uptake and Efflux Studies

Cell uptake and efflux studies of $^{64}CuCl_2$ were performed on B16F10 and A375M cells. Briefly, melanoma cells (0.3×10^6 per well, n=3 for each group) were plated in 12-well plates and incubated at 37°C overnight. The cells were then incubated for various times (0.5, 1, 2 and 4 h) at 37°C with 1 μCi (37 KBq) of $^{64}CuCl_2$ in serum-free medium. Moreover, non-radioactive $CuCl_2$ (20 nmol/mL, 0.5 mL per well) was also used to block the uptake of $^{64}CuCl_2$ in another group of samples. At designated times, the radioactive medium was aspirated, and cells were washed 3 times with ice-cold PBS before being lysed with 0.1% SDS-0.02 M NaOH for 5 min at room temperature and finally being rinsed 3 times with PBS. The radioactivity of the cell lysates was counted by a Wallac 1480 automated γ-counter (PerkinElmer, Waltham, MA, USA). The counts per minute and percentage of uptake were plotted as a function of time using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). For efflux studies, the cells were initially incubated with $^{64}CuCl_2$ for 2 h under the conditions described above. Then, the

radioactive medium was aspirated, and the cells were washed 3 times with PBS. Fresh medium was added, and the cells were maintained at 37°C. At a different time (0.5, 1, 2, 4 and 16 h), the supernatant and cell lysate were collected separately when their radioactivity was counted. Cellular retention was calculated by dividing the radioactivity of the cells by the total radioactivity in the cells at time 0.

Small Animal PET, Biodistribution Studies and Radionuclide Therapy Study

Subcutaneous Tumor Model

Animal procedures were performed according to a protocol approved by the Stanford University Institutional Animal Care and Use Committee (IACUC). Female C57BL/6 mice and female athymic nude mice (nu/nu) were purchased from Charles River Laboratories (Boston, MA, USA) at 5–6 weeks old and kept under sterile conditions. Approximately 1×10^6 or 2×10^6 B16F10 cells suspended in 100 μL of PBS were implanted subcutaneously into the flanks of C57BL/6 mice. Nude mice were injected subcutaneously with 100 μL of PBS containing 5×10^6 A375M cells.

Small Animal PET, Biodistribution Studies

PET imaging was performed using a microPET R4 rodent model scanner (Siemens Medical Solutions USA, Inc., Knoxville, TN). For static scans, the mice bearing B16F10 or A375M tumors (n=4 for each group) were injected with $^{64}CuCl_2$ via the tail vein (85.6~88.5 μCi). At different times (1, 2, 4, 24, 48, 72 h) post-injection (p.i.), the mice were anesthetized with isoflurane (5% for induction and 2% for maintenance in 100% O_2) and were placed in the prone position near the center of the field of view of the scanner, and 5-min static scans were then obtained. Dynamic scans of B16F10 tumor–bearing mice were started 2 min after tail vein

injection of $^{64}CuCl_2$ and continued for 35 min. Moreover, for a direct comparison of uptake of free $^{64}CuCl_2$ in liver versus unstable demetallated ^{64}Cu from DOTA, $^{64}CuCl_2$ or ^{64}Cu-DOTA (~100 μCi) was injected into nude mice (n=3) and static PET imaging was performed at 12, 24, 48 and 72 h p.i. The images were reconstructed by a two-dimensional ordered subsets-expectation maximum (OSEM) algorithm. Regions of interest (ROIs) were then drawn over the tumor and other important organs on decay-corrected whole-body coronal images. The mean counts per pixel per minute were obtained from the ROIs and converted to counts per milliliter per minute by using a calibration constant. By assuming a tissue density of 1 g/mL, the ROIs were converted to counts/g/min. An image ROI-derived percentage of the injected radioactive dose per gram of tissue (%ID/g) was then determined by dividing the counts per gram per minute with the injected dose (ID). No attenuation correction was performed.

Biodistribution studies were conducted after the 72 h PET imaging was finished. The mice were sacrificed; the tumors and normal tissues of interest (blood, heart, lung, liver, spleen, pancreas, stomach, intestine, kidneys, brain, skin, muscle and bone) were removed and weighed; and the radioactivity was measured by the γ-counter. The radioactivity uptake was expressed as %ID/g.

Radionuclide Therapy Study

Mice bearing B16F10 or A375M tumors were subjected to $^{64}CuCl_2$ treatment when tumors reached 0.5–0.8 cm in diameter. The mice were randomly divided into treatment groups and control groups (see Supplemental Table 1). 0.15 mL of $^{64}CuCl_2$ or PBS (control group) was administered via the tail vein. The weights of the mice and tumor size were measured daily for B16F10-bearing mice and every 2~3 days for A375M-bearing mice. Tumor volumes were

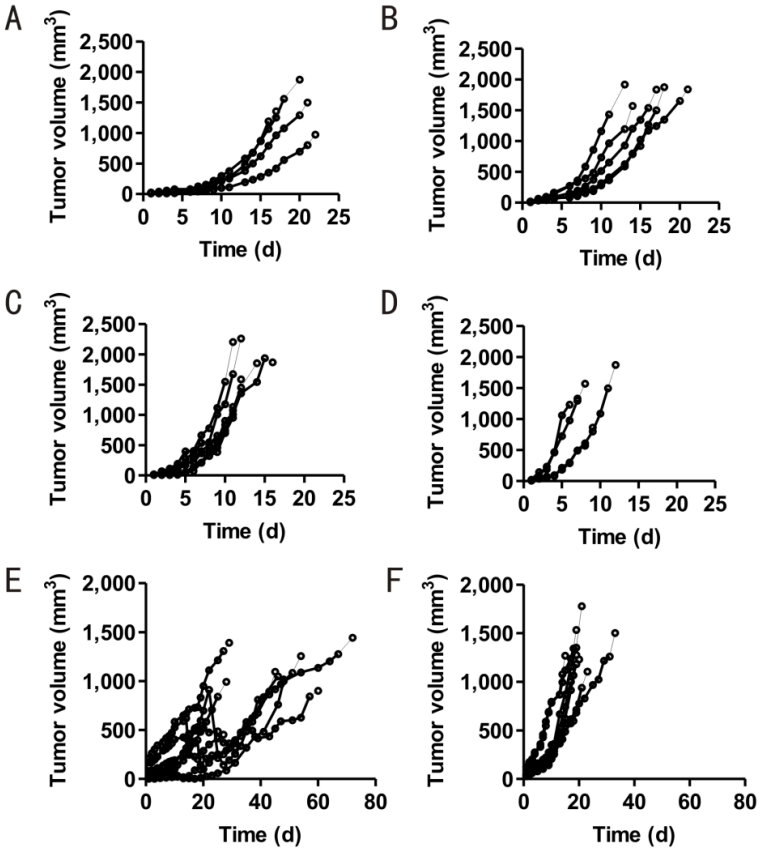
calculated by the following formula: volume = $(\text{width})^2 \times \text{length}/2$. The mice were sacrificed when the tumor was >1.75 cm in diameter, or >10% of the original body weight, was preventing normal locomotion, or was ulcerated or when the animal was moribund (according to the guidelines).

SUPPLEMENTAL TABLE 1 Groups of mice used in the study. Different tumor models were prepared by subcutaneous injection of different melanoma cell types and cell numbers.

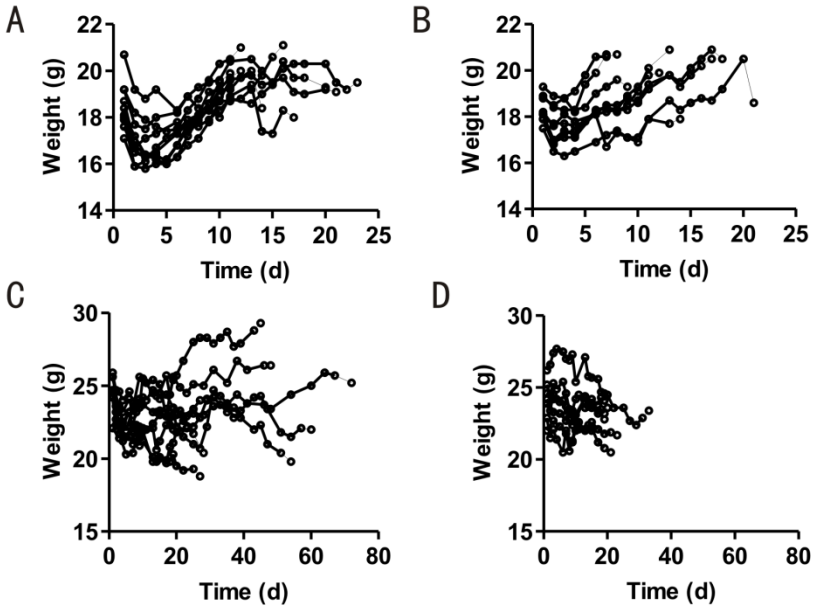
Cell type	Cell numbers	Therapy groups			Control groups		
		Group No.	Group size	$^{64}CuCl_2$ Dosage (mCi/mouse)	Group No.	Group size	$^{64}CuCl_2$ Dosage (mCi/mouse)
B16F10	1×10^6	1	n=4	2.11 ± 0.15	4	n=5	0 (PBS)
B16F10	2×10^6	2	n=6		5	n=4	
A375M	5×10^6	3	n=8	2.25 ± 0.03	6	n=7	0 (PBS)

RESULTS

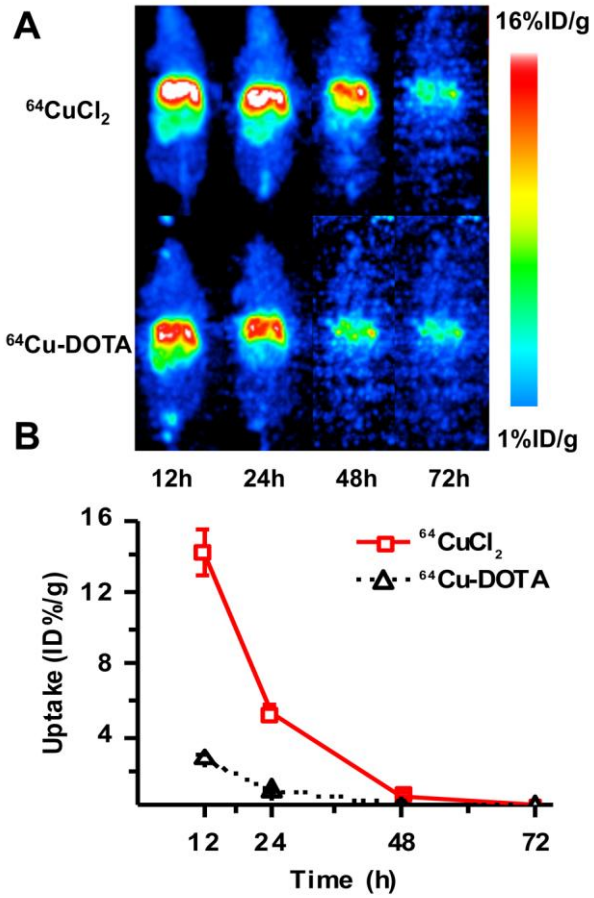
Radionuclide Therapy



Supplemental Figure 1. Tumor growth curves of the $^{64}CuCl_2$ therapy group (A, C and E) and the PBS control group (B, D, and F) in B16F10 (A~D) and A375M (E and F) tumor models. **A** and **B** represent B16F10 models injected with 1 million cells. **C** and **D** represent B16F10 models implanted with 2 million cells. Tumor volumes are expressed in mm^3; and time is expressed in days from therapy.



Supplemental Figure 2. Weight variation curves of the $^{64}CuCl_2$ therapy group (A and C) and the PBS control group (B and D) in B16F10 (A and B) and A375M (C and D) tumor models. Weight is expressed in g; and time is expressed in days from therapy.



Supplemental Figure 3. A. Decay-corrected whole-body coronal microPET images of nude mice from 5-min static scans at 12, 24, 48 and 72 h after intravenous injection of $^{64}CuCl_2$ **(upper row)** or ^{64}Cu-DOTA **(lower row**). **B** MicroPET quantification of liver uptake after the intravenous injection of $^{64}CuCl_2$ or ^{64}Cu-DOTA, respectively (n = 3).