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Supporting Information

Hyperfluorescence Imaging of Kidney Cancer Enabled by Renal Secretion Pathway Dependent Efflux Transport

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Abstract: Renal tubular secretion is an active efflux pathway for the kidneys to remove molecules but has yet to be used to enhance kidney cancer targeting. We report indocyanine green (ICG) conjugated with a 2100Da PEG molecule (ICG-PEG45) as a renal-tubule-secreted near-infrared-emitting fluorophore for hyperfluorescence imaging of kidney cancers, which cannot be achieved with hepatobiliary- and glomerular-clearable ICG. This pathway-dependent targeting of kidney cancer arises from the fact that the secretion pathway enables ICG-PEG45 to be effectively effluxed out of normal proximal tubules through P-glycoprotein transporter while being retained in cancerous kidney tissues with low P-glycoprotein expression. Tuning elimination pathways and utilizing different efflux kinetics of medical agents in normal and diseased tissues could be a new strategy for tackling challenges in disease diagnosis and treatments that cannot be addressed with passive and ligand-receptor-mediated active targeting.

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Experimental Procedures

Materials and equipment

PEG samples with average molecular weight of 1100 Da, 2100 Da, 3500 Da, 5000 Da and 10100 Da were purchased from Sigma-Aldrich (USA). ICG-NHS and IRDye800CW-NHS were purchased from Intrace Medical (Switzerland) and LI-COR, respectively. Absorption spectra were measured by a Virian 50 Bio UV-vis spectrophotometer. Fluorescence spectra were acquired by a PTI QuantaMaster™ 30 Fluorescence Spectrophotomer (Birmingham, NJ). In vivo fluorescence images were recorded using a Carestream In-vivo FX Pro imaging system. Optical images of cultured cells and tissue slides were obtained with an Olympus IX-71 inverted fluorescence microscope coupled with Photon Max 512 CCD camera (Princeton Instruments). Agarose gel electrophoresis was carried out by a Bio-Rad Mini-Sub Cell GT system. Animal studies were performed according to the guidelines of the University of Texas System Institutional Animal Care and Use Committee. BALB/c mice (BALB/cAnNCr, strain code 047) of 6-8 weeks old, weighing 20-25g, were purchased from Envigo. Nude mice (Athymic NCr-nu/nu, strain code 069) of 6-8 weeks old, weighing 20-25 g, were also purchased from Envigo. All of these mice were randomly allocated and housed under standard environmental conditions (23±1 ℃, 50±5% humidity and a 12/12 h light/dark cycle) with free access to water and standard laboratory food.

Synthesis of ICG-PEG conjugates

400 μL, 10 mM PEG molecule in ultrapure water was added into 400 μL, 400 μM ICG-NHS in DMSO and the mixture was vortexed for 3 h. Then ICG-PEG conjugates were purified with sephadex column from unconjugated ICG and PEG molecule with mobile phase of ultrapure water. The different mobilities of ICG, ICG-PEG22, ICG-PEG45 and ICG-PEG220 in sephadex column and agarose gel both proved the successful synthesis of ICG-PEG conjugates. The IRDye800CW-PEG45 is synthesized in a similar way with ICG-PEG45 and detailed procedures were reported previously[1].

Serum protein binding test

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To test whether the PEG conjugation will affect the protein binding of ICG or not, free ICG, ICG-PEG22, ICG-PEG45 and ICG-PEG220 were incubated with either phosphate-buffered saline (PBS) or PBS supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 ℃ for 30 min. In order to identify the colorless protein band, FBS incubated ICG and ICG-PEGn were stained by 10% (v/v) Coomassie Brilliant Blue 250 (CBB 250). All these samples were analyzed using 2% agarose gel electrophoresis. In addition to gel electrophoresis, the protein binding of ICG and ICG-PEGn was also tested by quantifying their fluorescence intensity in water and in 100% FBS (same concentration, but different solvent), as shown in Fig. S6. The fluorescence images were taken by Carestream In-vivo FX Pro imaging system under 790nm/830nm.

Non-invasive fluorescence imaging of body with ICG-PEG conjugates and ex vivo images

Hair-removed BALB/c mouse (~25 g/mouse) was prone-positioned on imaging stage of Carestream In-vivo FX Pro imaging system under 3% isoflurane anesthesia and then intravenously injected by 200 μL, 40 μM ICG or ICG-PEG conjugates, following a start of sequential time-series imaging collection for 10 min. The fluorescence imaging parameters were set as follow: EX760/EM830 nm; 10 sec exposure time; 2×2 binning. At 10 min post injection, liver, kidneys, heart, spleen and urine in bladder were harvested and were imaged under EX790/EM830 nm, 10 sec exposure.

Clearance efficiencies of ICG-PEG conjugates in urine and feces

BALB/c mice were intravenously injected with ICG (n=3), ICG-PEG conjugates (n=3 for each conjugate), respectively, with concentration of 40 μM and injection volume of 200 μL and then placed in metabolism cages. The separated mouse urine and feces from ICG, ICG-PEG22, ICG-PEG45 and ICG-PEG220 were collected at 24 h post injection. Then, the urine and feces were quantified based on fluorescence and each standard curve of conjugates were built in control urine and control feces.

Noninvasive fluorescence imaging of kidney with ICG-PEG45

The hair-removed BALB/c mouse was anesthetized using 3% isoflurane and a catheter filled with PBS was inserted into the tail vein. The mouse with tail vein catheter was placed in supine position on the imaging stage of Carestream In-vivo FX Pro imaging system, allowing the back to face the excitation light and CCD camera. Mouse with the steady breath rate of 10-14 times per 15 sec was injected PBS solution of ICG-PEG45 (200 μL, 40 μM) and then followed sequential time-series imaging (10 sec exposure) collection with EX790/EM830 nm for ICG-PEG45.

Kidney slides imaging with optical microcopy

BALB/c mice were sacrificed at 5 min, 10 min and 1 h after intravenous administration of 200 μL, 400 μM of ICG-PEG45. And other BALB/c mice were sacrificed at 5 min and 1 h after intravenous administration of 200 μL, 400 μM of 800CW-PEG45. The kidneys were then collected and fixed immediately in 10% neutral buffered formalin, followed by standard dehydration and paraffin embedding. The embedded tissues were then sectioned into 4 μm slices and H&E stained. The final slides were visualized under Olympus IX-71 fluorescence microscope. The filters used for ICG-PEG45 are EX775/EM845 and dichroic mirror 810 nm. The filters use for 800CW-PEG45 are EX747/EM780LP and dichroic mirror 776 nm.

Probenecid inhibition studies

BALB/c mice were pre-treated by intraperitoneal injection of 200 mg/kg probenecid and control group were treated by PBS. After 30 min, ICG-PEG45, 800CW-PEG45 and FITC-inulin were intravenously injected into control group of mice and probenecid-treated mice, respectively, n=3 for each group. The mice were anesthetized under 3% isoflurane for 30 min under steady breath rate of 7-8 times per 15 sec. After 30 min, the urine was collected from bladder and quantified by fluorescence.

RCC and RCC metastasis model set-up

Orthotopic model (i.e., sponge model): orthotopic model was established by performing unilateral renal implantation[2]. Briefly, the procedures were performed by a 1.0-cm dorsal incision using 6-8 weeks old NOD/SCID mouse. The cell suspension (1-2×10^6 cells) mixed with Gelfoam ®(Ethicon, Somerville, NJ) was implanted into subcapsular space of the left kidney with a capillary tube. Tumor growth was monitored by Bioluminescent imaging using IVIS® Spectrum (PerkinElmer, Waltham, MA). PDX (patients derived xenograft) model was used frozen RCC-PDX tissue samples (20-30 mm^3) from UT Southwestern Kidney Cancer and SPORE program. Tissues were surgically implanted into left kidney of 6-8 weeks-old male NOD/SCID mice as previously described[3]. Metastatic tumor models were established by intravenously injection of RCC cells through tail vein into 6-8 weeks-old male NOD/SCID mice. Tumors were monitored by Bioluminescent imaging. All experimental procedures were approved by the Institutional Animal Care and Use Committee. When above tumors were ready, 40 μM, 200 μL ICG-PEG45 were intravenously injected into mice. Normal right kidney and left kidney with RCC were collected and imaged at 24 h (orthotopic model), 68 h (PDX) after injection of ICG-PEG45. Metastatic tumors were collected and imaged at 24 h after injection of ICG-PEG45.

Noninvasive fluorescence imaging of RCC-implanted kidney with ICG-PEG45

The hair-removed RCC-implanted NOD/SCID mouse was anesthetized using 3% isoflurane and a catheter filled with PBS was inserted into the tail vein. The mouse with tail vein catheter was placed in supine position on the imaging stage of Carestream In-vivo FX Pro imaging system, allowing the back to face the excitation light and CCD camera. The mouse was imaged at 30 min, 1 h, 3 h, 5 h, 12 h and 24 h after injection of ICG-PEG45 (200 μL, 40 μM) under EX790/EM830 nm (10 sec exposure).

Ex vivo RCC fluorescent imaging

Orthotopic papillary RCC-implanted NOD/SCID mouse was sacrificed at 24 h (PDX ccRCC, 68 h) after intravenous injection of ICG-PEG45. The RCC-implanted left kidney and normal right kidney were both collected and cut into half for ex vivo fluorescent imaging under EX790/EM830 nm. Then the left RCC-implanted kidney was fixed immediately in 10% neutral buffered formalin, followed by standard dehydration and paraffin embedding. The embedded tissues were then sectioned into 4 μm slices and H&E stained. The final slides were visualized under Olympus IX-71 fluorescence microscope. The filters used for ICG-PEG45 are EX775/EM845 and dichroic mirror 810 nm.

P-gP expression measurement by western blot

The papillary renal cell carcinoma cell (ACHN), normal kidney proximal tubular cell (HK2), normal kidney tissue and RCC tissue (ACHN tumor) were lysed using radioimmunoprecipitation assay buffer (RIPA buffer, 150 mM NaCl, 1% Triton X-100, 50 mM Tris pH 8.0, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate containing 1% protease inhibitor cocktail [Roche, Indianapolis, IN]) and the same amount (30 μg) of protein from each sample was electrophoresized on 4-12% gradient Bolt gels (Life Technologies) then electroblotted onto nitrocellulose membranes. The membrane was incubated with 5% nonfat dry milk (w/v) for 1 h and then washed in PBS containing 0.1% Tween-20. Membranes were then incubated with designed primary monoclonal antibody (MA1-26528, Invitrogen, Carlsbad, CA) and the corresponding secondary antibodies conjugated with horseradish peroxidase (HRP) at room temperature for 1 h. The target proteins (P-gP) were detected with a fluorChem digital imaging system (Alpha Innotech, San Leandro, CA) using Western Bright Quantum HPR Substrate Kit (Advansta, Menlo Park, CA). Actin was used as an internal loading control for the measurement of P-gP expression at the cellular level.

Cellular uptake of ICG-PEG45 by HK2 and ACHN before and after inhibitor treatment

The papillary renal cell carcinoma cell (ACHN), normal kidney proximal tubular cell (HK2) (1 x 10^5) were seeded in peri-dish (n=3) and allowed to adhere and grow for 24 h. These cells were incubated with 25 μM ICG-PEG45 with or without 20 μM cyclosporine A (Adipogen Life Sciences (San Diego, CA)) or 10 μM Tariquidar (MedChemexpress (Monmouth Junction, NJ)) for 1 hour in cell incubator. The same volume of DMSO was added as control. After 1 h incubation, the cells were washed 3 times with ice cold HEPES buffer solution and maintained with 1 mL of HEPES buffer solution for imaging. The cells were further imaged using fluorescent microscope with the ICG channel of excitation:710nm, emission:830nm. The exposure time were set as follow: 0.01s for bright field; 5s for fluorescent imaging. 4 images from 4 random areas were taken for each petri-dish. The average intensity of ICG inside each cell was quantified using ImageJ. The experiment was repeated 3 times with similar results.

Statistical analysis

Error bars are reported as mean ± s.d. The differences between groups were compared by analysis of Student's t-test. P-value <0.05 was considered to be statistically significant. N.S. means no significant difference with p value>0.05. Investigators conducting the experiments were not blinded.

Supplementary Figures

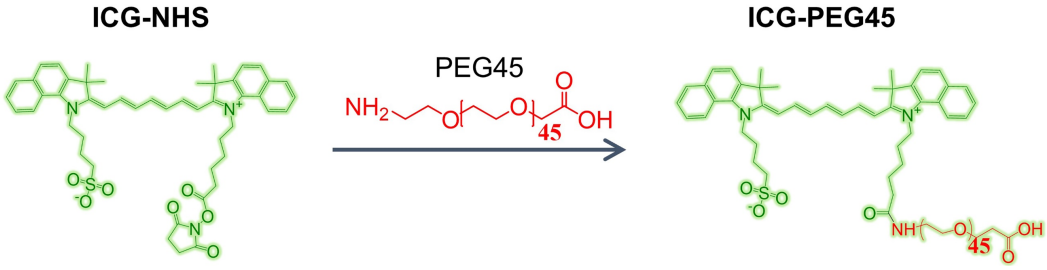
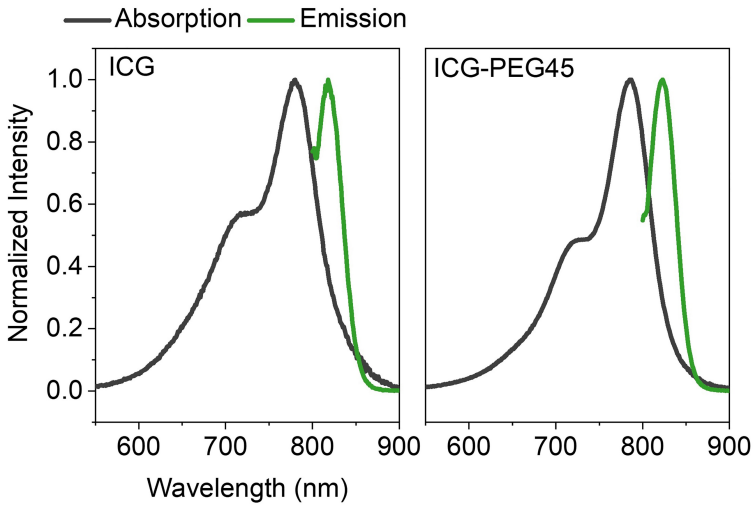


Figure S1. Synthesis of ICG-PEG45.

Figure S2. UV-vis absorption and fluorescence spectra of ICG and ICG-PEG45.



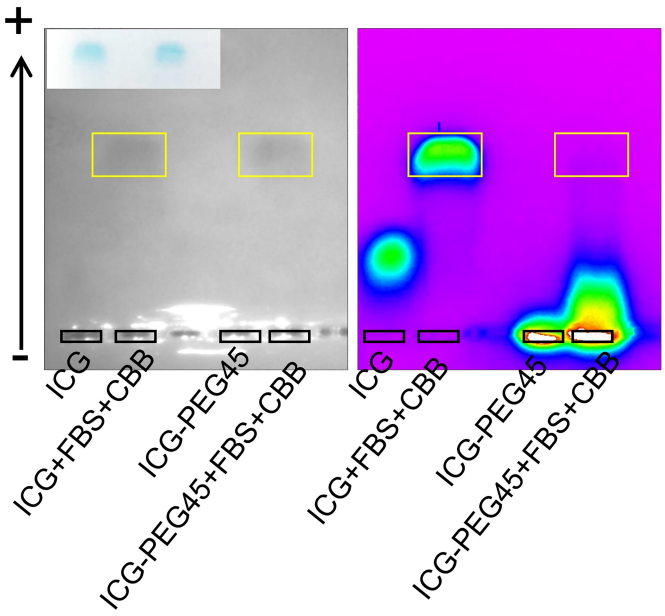


Figure S3. Serum protein binding test by agarose gel electrophoresis. The serum protein binding test of ICG and ICG-PEG45 was conducted by agarose gel electrophoresis in 10% fetal bovine serum (FBS) under 37 ℃ water bath for 30 min. Coomassie brilliant blue 250 (CBB) was used to stain FBS. Color pictures of CBB-stained protein were inserted. ICG completely binds protein, while conjugation of PEG45 molecules reduces its protein binding affinity.

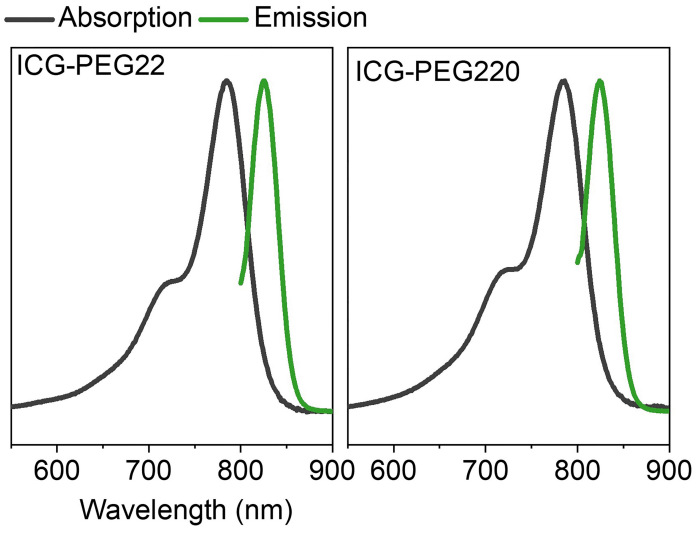


Figure S4. UV-vis absorption and fluorescence spectra of ICG-PEG22 and ICG-PEG220.

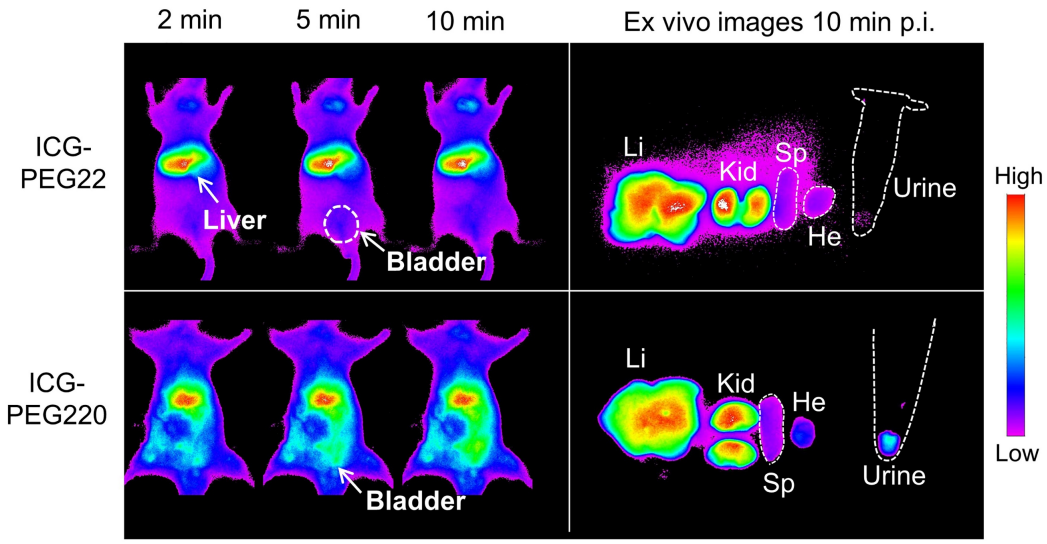


Figure S5. Real time *in vivo* imaging of ICG-PEG22 and ICG-PEG220 within 10 min post njection and ex vivo images of harvested organs. liver (Li), kidneys (Kid), heart (He), spleen (Sp) and urine collected from bladder at 10 min post intravenous injections (Ex/Em filters: 790/830 nm).

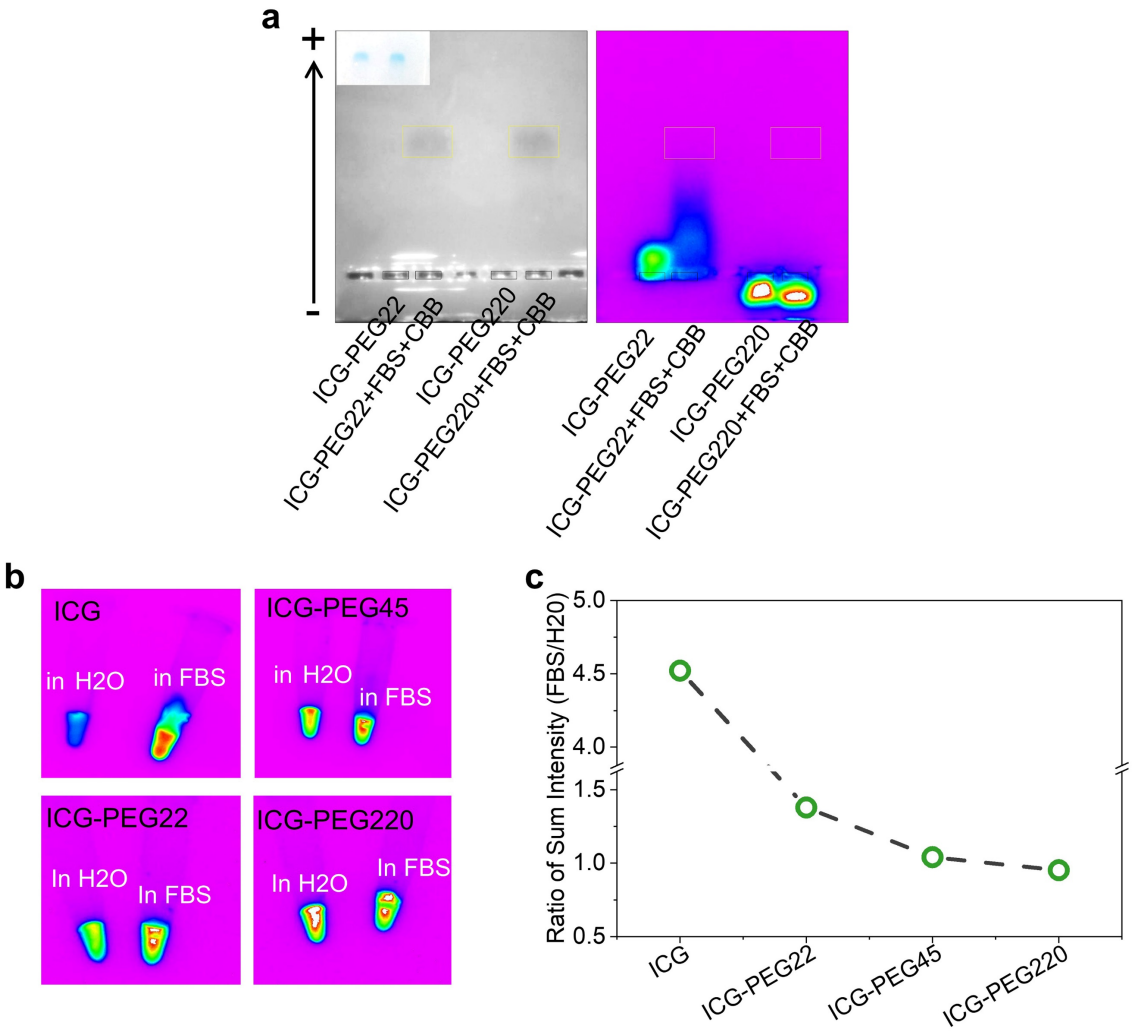


Figure S6. Serum protein binding. a, Serum protein binding test by agarose gel electrophoresis of ICG-PEG22 and ICG-PEG220 in 10% fetal bovine serum (FBS) under 37 ℃ water bath for 30 min. Coomassie brilliant blue 250 (CBB) was used to stain FBS. Color pictures of CBB-stained protein were inserted. b, Fluorescence intensity of ICG, ICG-PEG22, ICG-PEG45 and ICG-PEG220 in water and in 100% fetal bovine serum (FBS) (same dye concentration). c, The ratio of sum intensity in FBS over that in water of ICG, ICG-PEG22, ICG-PEG45 and ICG-PEG220. The ratio decreased with the increase of molecular weight of PEG, indicating that the protein binding of ICG is gradually decreasing with the increase of molecular weight of conjugated PEG molecules.

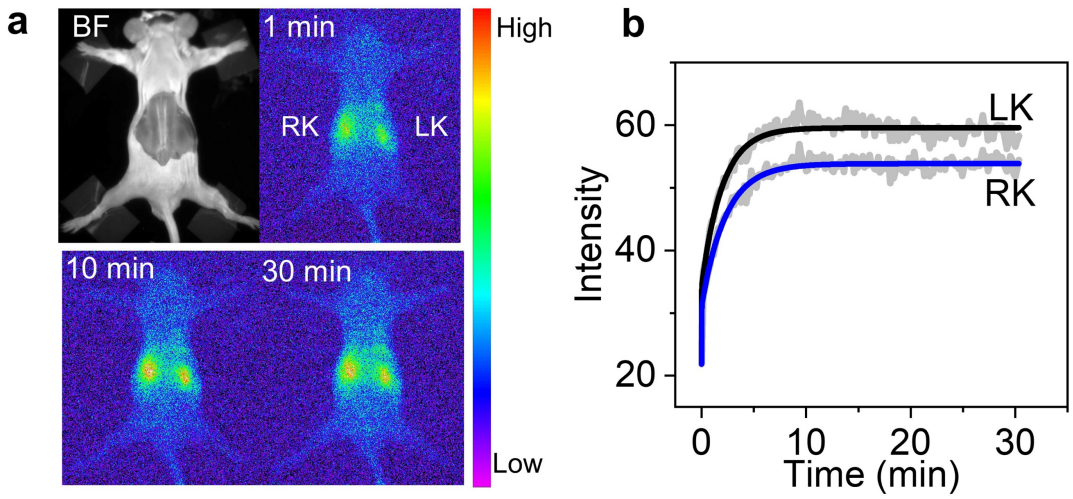


Figure S7. Kidney imaging of ICG-PEG45 with injection dosage of 4 μM. The concentration is 10 times lower than the dose used for ICG-PEG45 kidney imaging in the main text. The skin on the back side was removed to clearly monitor the fluorescence signals from kidneys. a. Bright field (BF) and fluorescence images at 1 min, 10 min and 30 min post intravenous injection of 4 μM ICG-PEG45. b. Kinetics of right kidney and left kidney over time. LK, left kidney. RK, right kidney.

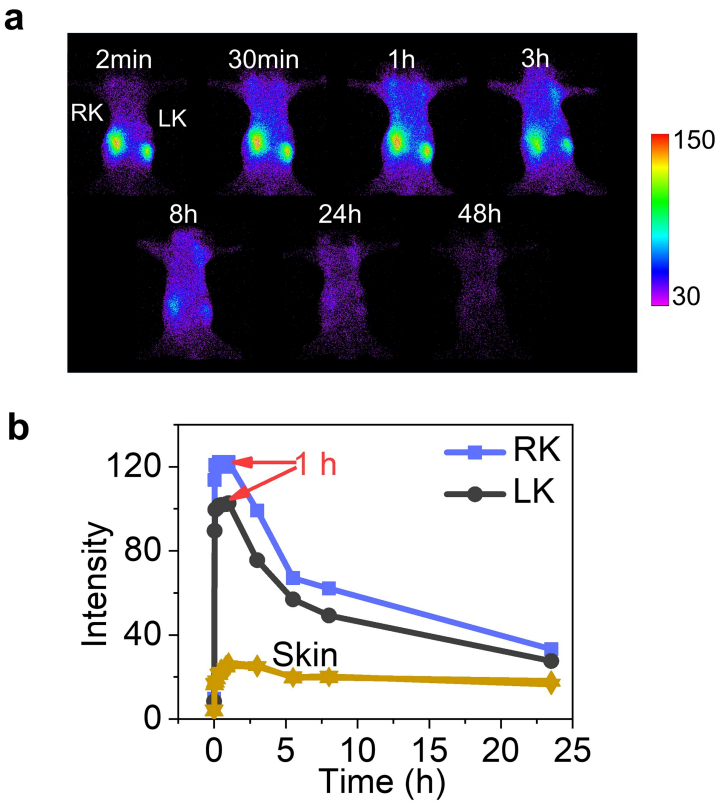


Figure S8. Long-term non-invasive kidney imaging of ICG-PEG45. a. Real time non-invasive kidney imaging post injection of ICG-PEG45. b. Kinetics curves of right kidney (RK), left kidney (LK) and skin over time.

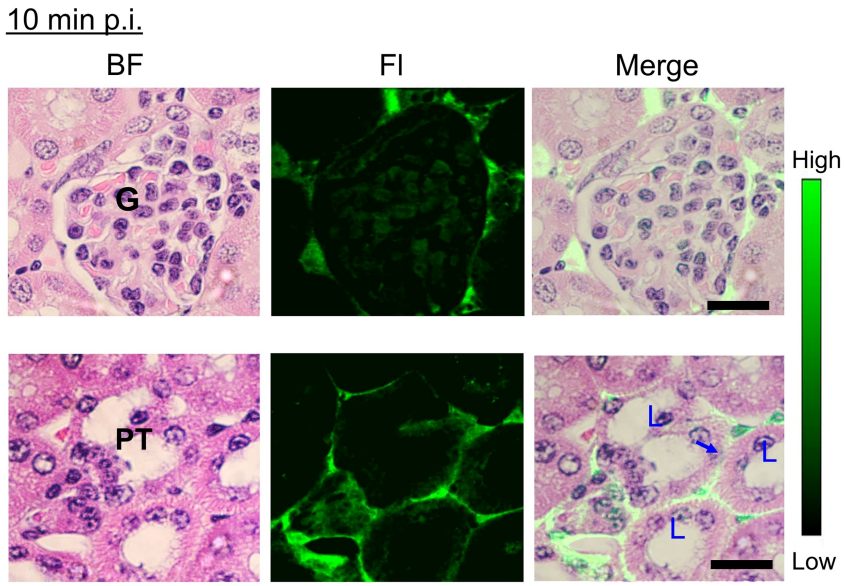


Figure S9. The distribution of ICG-PEG45 in glomerulus and tubules at 10 min post intravenously injection (p.i.). BF, bright field. FI, fluorescence. Scale bar is 20 μm. G, glomerulus, PT, proximal tubule, L, lumen.

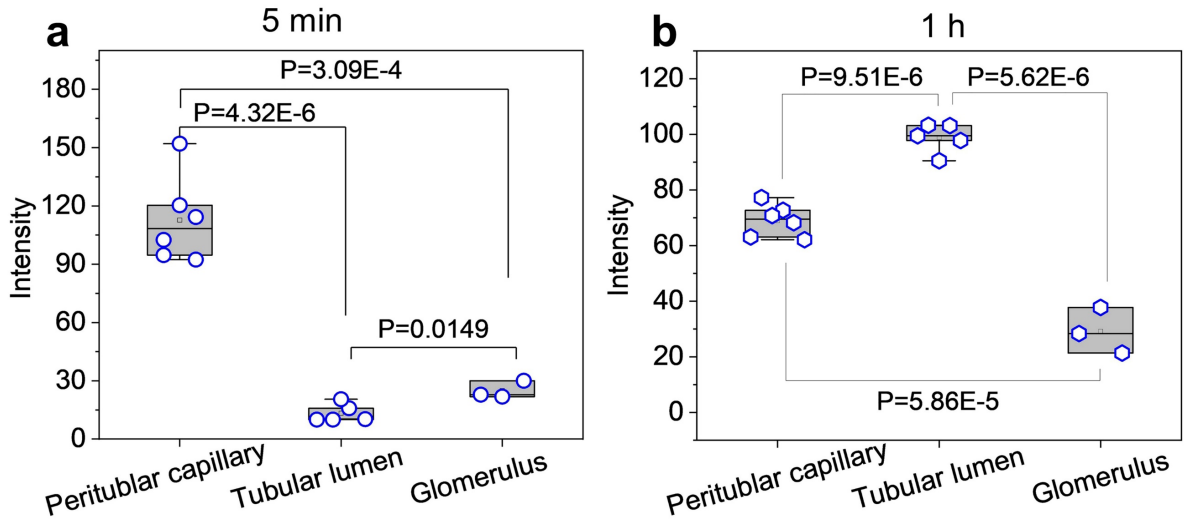


Figure S10. The signal intensity of ICG-PEG45 in different compartments of the kidneys. a, The signal intensity of ICG-PEG45 in peritubular capillary, tubular lumen and glomerulus at 5 min post injection. b, The signal intensity of ICG-PEG45 in peritubular capillary, tubular lumen and glomerulus at 1 h post injection. The p value is calculated based on student t-test.

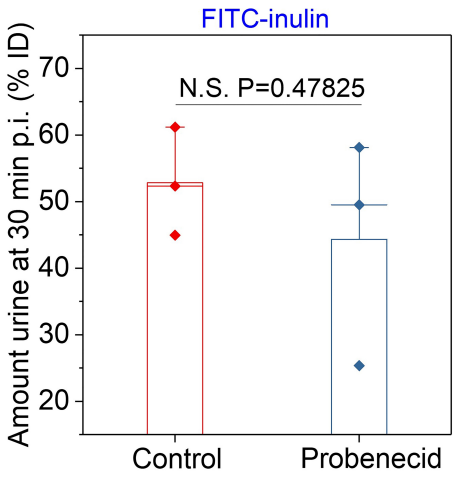


Figure S11. The effect of probenecid on the renal clearance efficiency of FITC-inulin. n=3. N.S. means no significant difference based on Student-t test, P>0.05.

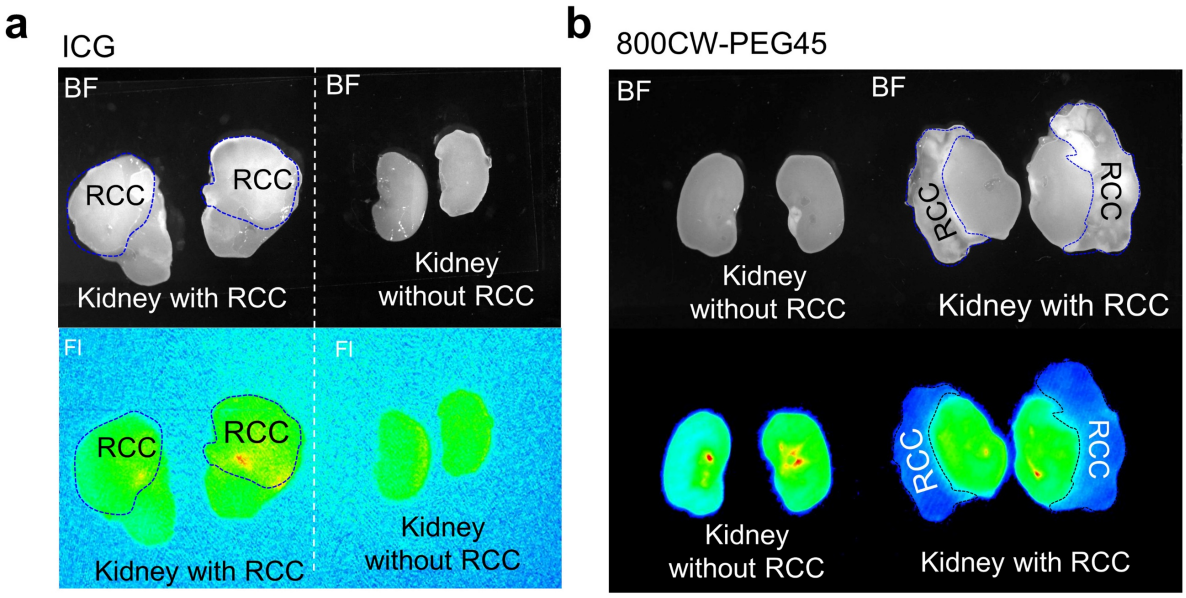


Figure S12. Ex vivo fluorescent images of kidneys with RCC and without RCC after injection of ICG (**a**) and 800CW-PEG45 (**b**), respectively, at 24 h. BF, bright filed. FI, fluorescence. RCC, renal cell carcinoma.

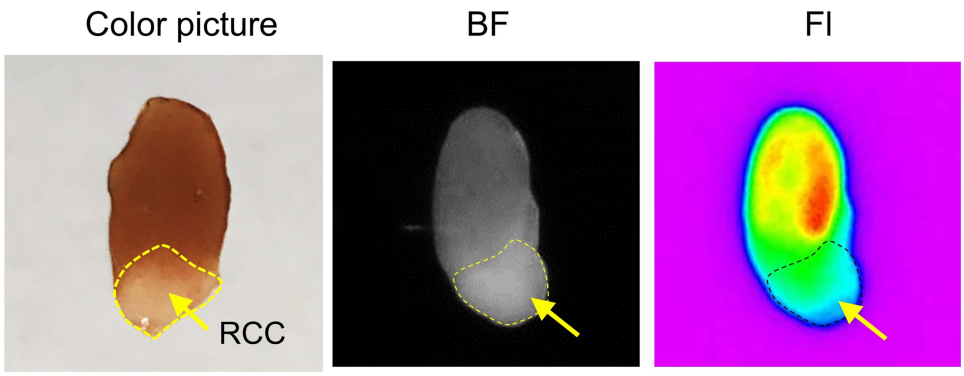
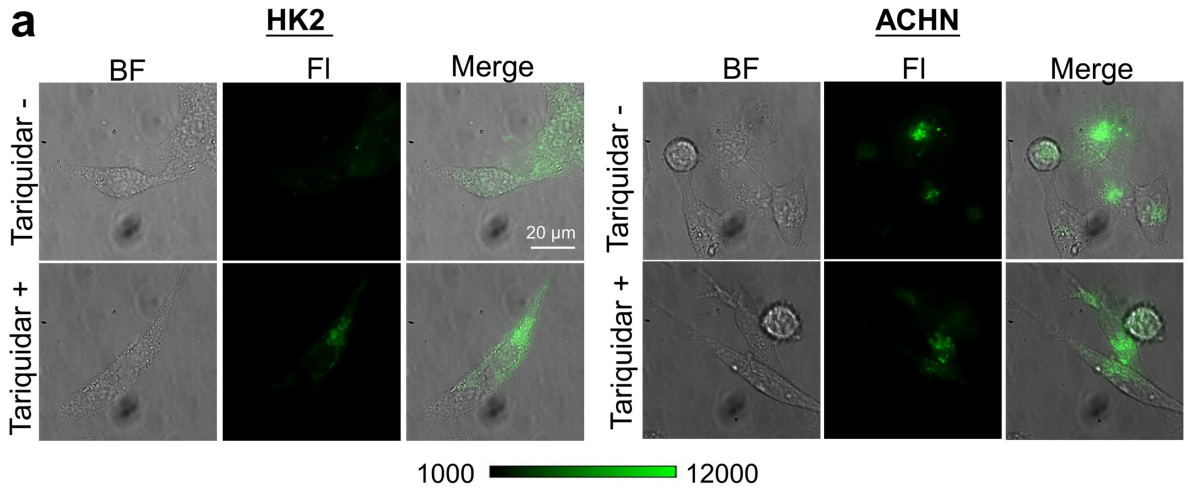


Figure S13. Ex vivo RCC at 24 h post intravenous injection of ICG-Au25. RCC, renal cell carcinoma. BF, bright field. FI, fluorescence.



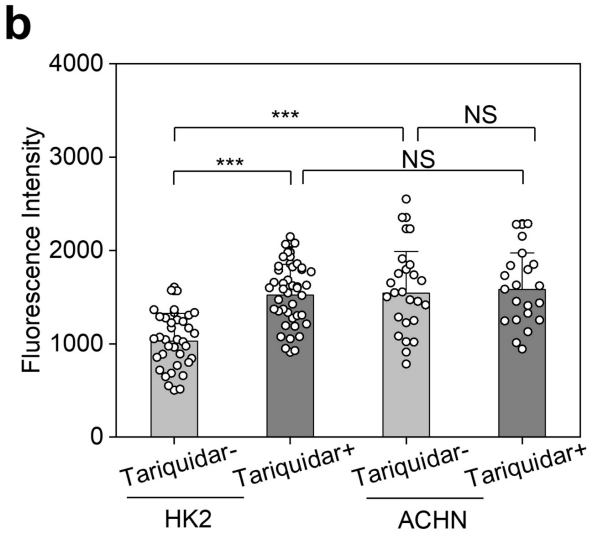


Figure S14. The cellular uptake of ICG-PEG45 under tariquidar treatment. **a,** The cellular uptake fluorescence imaging of ICG-PEG45 in HK2 and ACHN before and after the tariquidar treatment. scalar bar is 20 μm. BF, bright field. FI, fluorescence. **b.** The quantification of fluorescence intensity of ICG-PEG45. *** represents statistically different based on student t-test and P<0.0005. N.S. represents no significant difference based on student-test, P>0.05.

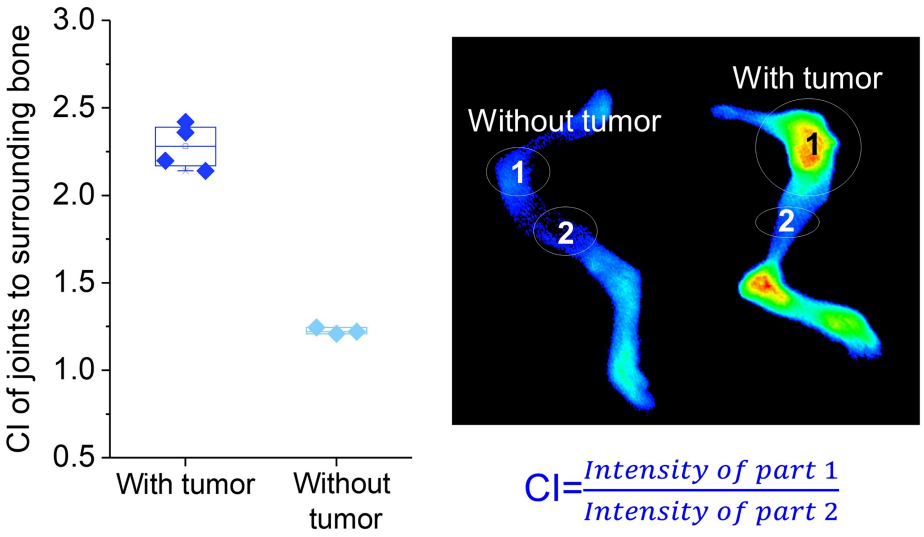


Figure S15. Contrast index of joints with tumor and normal joints of mice after injection of ICG-PEG45 at 24 h. The contrast index (CI) is calculated by the intensity of part 1 over part 2 (circled in figures).

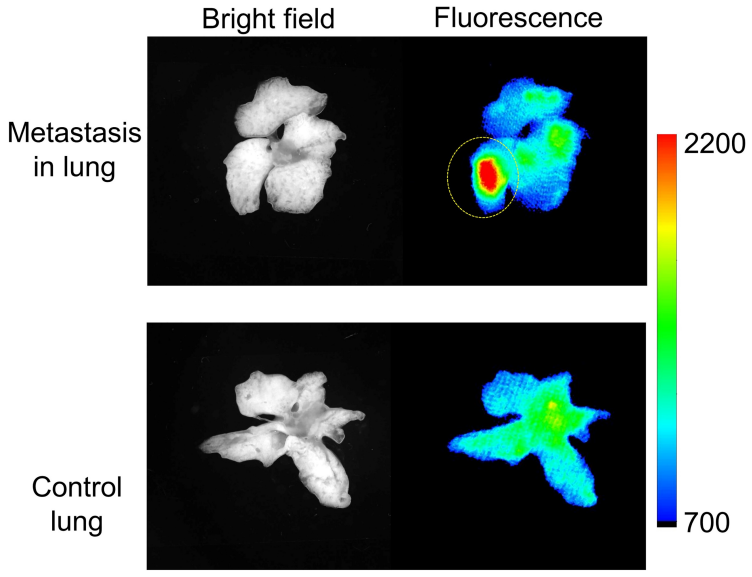


Figure S16. Ex vivo fluorescent images of renal cell carcinoma metastasis in lung by ICG-PEG45 at 24 h post intravenous injection.

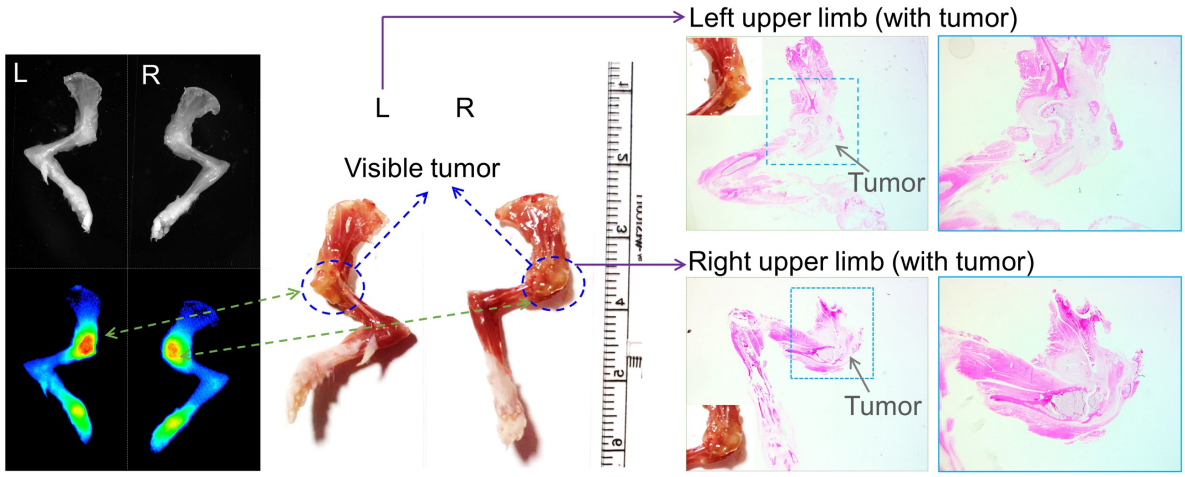


Figure S17. Ex vivo images and color images of tumor-bearing upper limbs from mouse at 24 h post injection of ICG-PEG45 and H&E pathology images.

References

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[3] A. Pavía-Jiménez, V. T. Tcheuyap, J. Brugarolas, *Nature protocols* **2014**, *9*, 1848.

Author Contributions

J.Z. conceived the idea and designed the experiments with B.D. B.D. conducted the experiments with the assistance of X.J. and J.L., Y.C. conducted the in vitro studies with the assistance of S.L. U.L. and Y.C. conducted the quantification of P-gP expression level by western blot. RCC and associated metastasis models were provided by J.H. under the help of A.D. Y.C. and E.H.. B.D. discussed and analyzed the results with J.Z. and M.Y. J.Z., M.Y. and B.D. composed the manuscript. All authors commented on the manuscript.