Supplementary Material

For

Transforming a targeted porphyrin theranostic agent into a PET imaging probe for cancer

By J Shi, TW Liu, J Chen, D Green, D Jaffray, BC Wilson, F Wang and G Zheng

Experimental Section

^{64}Cu-Radiolabeling: In a 1.5 mL eppendorf tube, 2 μL DMSO was added to dissolve 50 μg (~30 nmol) of PPF (Pyro-Peptide-Folate). 0.1 mL of 0.1 M NH_4OAc buffer (pH = 5.5) was added and vortexed, producing a dark green solution. 0.10 mL of $^{64}Cu(Acetate)_2$ solution (0.5 - 5.0 mCi) was then added and the reaction mixture was heated in a water bath at 60^{o}C for 20 min. After cooling to room temperature, a sample of resulting solution was analyzed by radio-UPLC.

The radiolabeling yield was > 99.9% and the radiochemical purity of ^{64}Cu-PPF was > 98% (this depends on the purity of the starting material PPF) and the specific activity was 2.66×10^6 GBq/mol.

The radio-UPLC method: The radio-UPLC method used the Acquity^{TM} Waters UPLC system (Waters Corp., Milford, MA) equipped with PDA detector and Bioscan radioactive detector and Acquity BEH C18 column (2.1 × 100 mm, 1.7μm; Waters). The flow rate was 0.8 mL/min. The mobile phase was isocratic with 80% solvent A (0.1 M TEAA, pH 7) and 20% solvent B (acetonitrile) at 0 min, followed by a gradient mobile

phase shifting from 20% solvent B at 0 min to 100% solvent B at 12 min and back to 20% solvent B at 13-15 min.

Purification: Purification with Sep-Pak C18 cartridge was done according to the following procedure: 1) Attach a syringe to the Sep-Pak C18 cartridge. 2) Flush the column with 5 mL of ethanol and flush the column with 10 mL of saline to equilibrate the column. 3) Load the sample onto the column and wash the sample with 10 mL of saline. 4) Elute with 400 μl of 80% ethanol, collect the fractions of purified sample. 5) Dry samples using a speed-vacuum and resuspend in saline. A certain amount of radioactive is washed down in step 3 if unlabeled free ^{64}Cu is observed in the system. With the natural dark green color of Pyro, the elution of Pyro-conjugate can be easily and directly monitored visually in step 4, and the fractions with the deepest color contain the highest concentration of labeled and unlabeled Pyro-conjugate.

Dose Preparation. ^{64}Cu-PPF was prepared and administered without any further purification. The dose solution was prepared by dissolving the radiotracer in saline to a concentration of 2.5 - 5.0 mCi/mL for MicroPET imaging, and diluted to a concentration of 0.1 - 0.5 mCi/mL. The resulting solution was filtered with a 0.20 μMillex-LG filter before being administered to the animals. Each tumor-bearing mouse was injected via the tail vein with 0.1 - 0.2 mL of the filtered dose solution.

Solution Stability: For *in vitro* solution stability studies, ^{64}Cu-PPF was prepared and used without any further purification. The ^{64}Cu-PPF was dissolved in a saline or serum solution (10% FBS in saline) with a final amount of 1 mCi/mL and left at room temperature for stability measurements. Samples of the resulting solution were analyzed

by radio-UPLC at 0, 6, and 24 h post-incubation. The samples from the serum solution were centrifuged before UPLC injection.

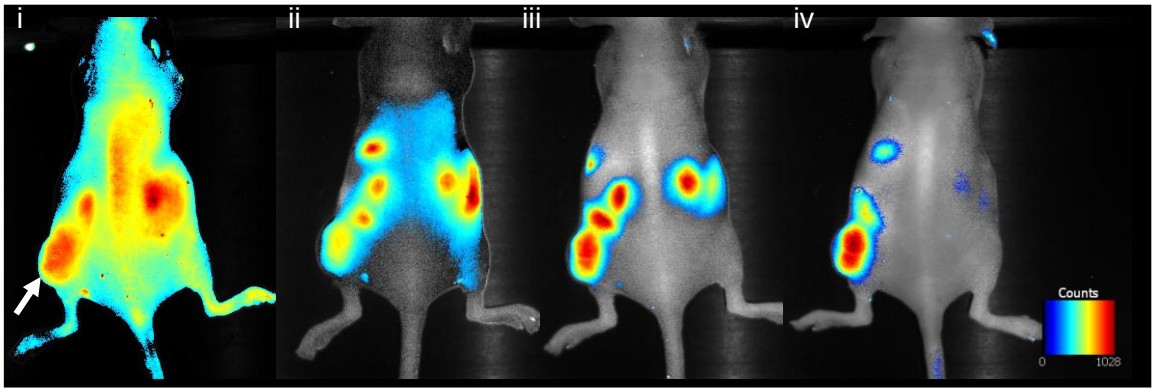
***In vivo* xenograft model:** All animal studies were carried out under institutional approval (University Health Network, Toronto, Canada). Adult athymic female nude mice were inoculated subcutaneously with $1x10^6$ of KB (FR-positive human epidermal cancer) or MT-1 (FR-positive human breast carcinoma) cells in 200μL of media in the left flank under general anesthesia with 2% isofluorane in oxygen. Animals were maintained in pathogen-free conditions in autoclaved microisolator cages. After 2 weeks, the tumors were 5-10 mm in diameter.

MicroPET/CT Imaging. MicroPET imaging was performed using a MicroPET R4 rodent model scanner (Concorde Microsystems, Knoxville, TN). The tumor-bearing mice (n = 3) were anesthetized with 2% isoflurane in oxygen, and injected with ~500 μCi of ^{64}Cu-PPF via the tail vein, and placed near the center of the FOV where the highest resolution and sensitivity are obtained. A 10-min static PET image was obtained at 4 h post injection and 30-45 min static PET images were acquired at 24 h post injection. Throughout the imaging, mice were kept anesthetized and directly transferred to the scanner, together with the supporting bed, without any movement. CT scanning was carried out immediately after each PET imaging session. For the blocking experiment, a mouse bearing a KB xenograft was injected with 500 μCi of ^{64}Cu-PPF along with 500-fold excess free folic acid. The static PET images were then acquired with same parameters at 4 and 24 h post injection.

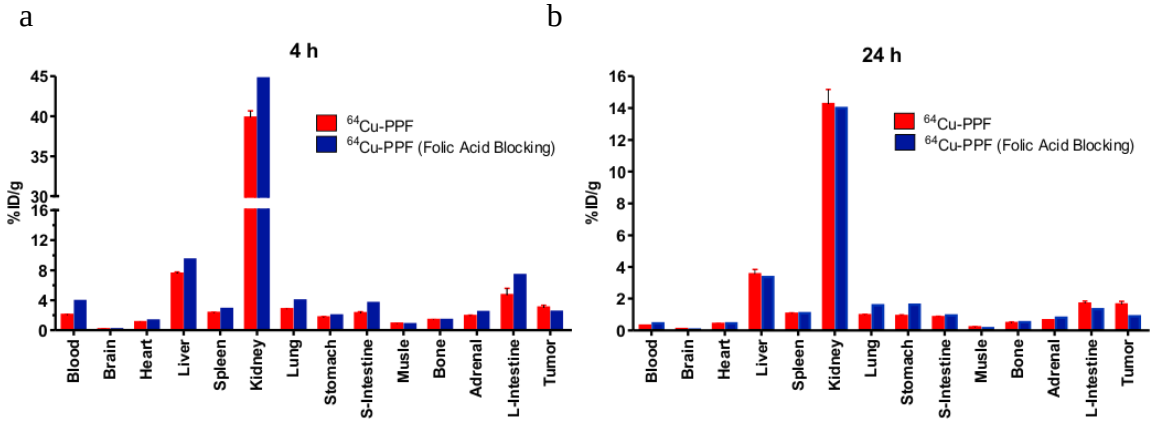
Biodistribution Studies: Biodistribution studies were performed using the athymic nude mice bearing KB xenografts. Twenty tumor-bearing mice (25 - 30 g) were randomly

divided into 5 groups. The ^{64}Cu radiotracer (~12.5 μCi in 0.1 mL saline) was administered into each animal via the tail vein. Four animals were euthanized by with 2% isoflurane, exsanguinationed, and opening of the thoracic cavity at 4 or 24 h post injection. Blood samples were withdrawn from the heart through a syringe. Organs were excised, washed with saline, dried with absorbent tissue, weighed and counted on a γ-counter (Perkin-Elmer Wizard-1480). Organs of interest included the tumor, heart, spleen, lungs, liver, kidneys, adrenal, stomach, intestine, muscle, bone and brain. Organ uptake was calculated as a percentage of the injected dose per gram of tissue (%ID/g). For the blocking experiment, each animal was administered with ~12.5 μCi of ^{64}Cu radiotracer along with more than 500-fold excess folic acid, and animals were sacrificed at 4 or 24 h post injection for biodistribution studies. The organ uptake (%ID/g) was compared to that obtained in the absence of excess folic acid at the same time point. The biodistribution data and target-to-background (T/B) ratios are reported as the mean and standard deviation based on results from three animals at each time point. Comparison between two different radiotracers was made using the two-way ANOVA test (GraphPad Prim 5.0, San Diego, CA). The level of significance was set at $p < 0.05$.

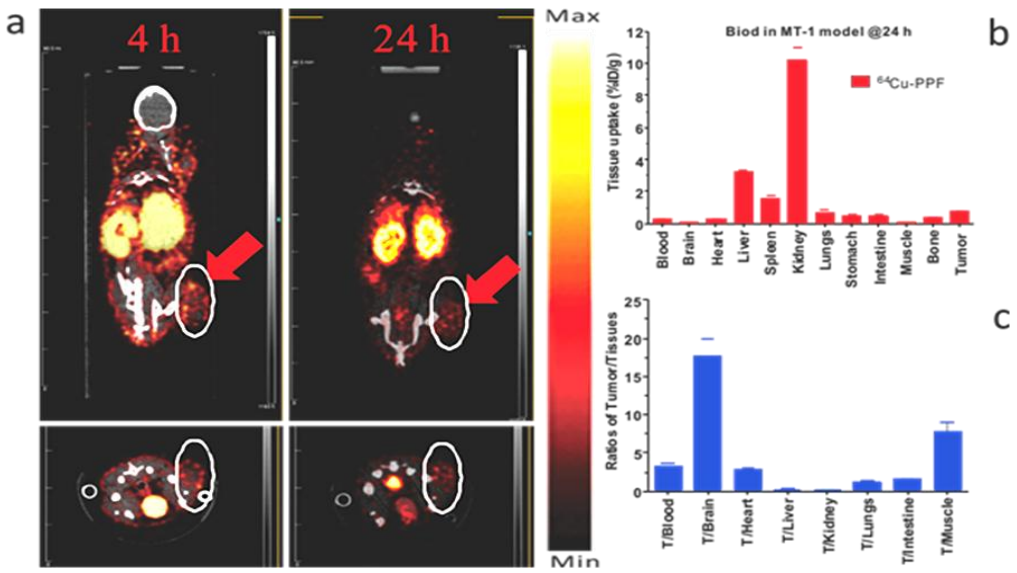
Metabolism: Normal athymic nude mice (n = 2) were used to evaluate the metabolic stability of ^{64}Cu-labeled PPF. Each mouse was injected with the ^{64}Cu radiotracer at a dose of ~200 μCi in 0.1 mL of saline via the tail vein. Urine samples were collected at 1.0 h post injection by manual void and mixed with saline solution. The mixture was centrifuged at 8,000 rpm for 3 min. The supernatant was collected and filtered through a 0.20 μm Millex-LG filter unit. The filtrate was analyzed by radio-UPLC.



Supplement Figure S1. Fluorescence images of tumor-bearing mice with KB (high FR expressing, white arrow) xenografts. Mice were administered 30nmol (in 200mL) of PPF and imaged i) 10 min, ii) 2h, iii) 6h, and iv) 24h post intravenous injection. At 24h, we observe favourable tumor uptake and biodistribution optically. This result was consistent with what demonstrated previously by Stefflova *et al.*[16]



Supplement Figure S2. a) Biodistribution of ^{64}Cu-PPF in selected organs (red bars) and 500 times excess Folic blocking (blue bars) at 4 h after intravenous injection. b) Biodistribution of ^{64}Cu-PPF in selected organs (red bars) and 500 times excess Folic blocking (blue bars) at 24 h post injection.



Supplement Figure S3. MicroPET/CT imaging and biodistribution of ^{64}Cu-PPF in MT-1 tumor model. a) Representative MicroPET/CT imaging (Coronal images (top) and single transverse slices passing through the tumors (bottom)) of MT-1 tumor bearing mice (n = 2) at 4, 24 h after intravenous injection of ^{64}Cu-PPF. b) Tissue uptake of ^{64}Cu-PPF, in selected organs at 24 h (red bars) after intravenous injection. c) Ratios of tumor-to-selected organs in mice administered with ^{64}Cu-PPF at 24 h (blue bars) post injection. Data are presented as mean ± SD (n = 2).