Supplemental Data

1. Synthesis and radiochemical synthesis

Chemicals were obtained from Aldrich, Alfa Aesar or Bachem unless otherwise stated. All solvents used were HPLC grade. TLC, where applicable, was performed on precoated aluminium-backed plates (Merck Kieselgel).

H_2ATSM was synthesised as previously reported (1, 2).

Radio-HPLC. Analytical reverse-phase HPLC spectra were recorded on a Gilson 322 instrument or a Dionex Ultimate 3000 system with a C-18 column (Grace Discovery Sciences, 250×4.6 mm, 5µ) and UV/vis detection at 254 nm. Solvent conditions were as follows. Solvent A = H_2O+0.1% TFA, solvent B = CH_3CN, flow rate = 1 mL min^{-1}, gradient (min, % of B): 0, 5; 20, 95; 25, 95; 28, 5; 30, 5.

Reverse-phase radio-HPLC (analytical and preparative) was performed on a Gilson 322 Unipoint HPLC machine equipped with the above C-18 column, UV/vis detection at 254 nm and NaI/PMT detection.

Copper-64. ^{64}Cu was purchased from the Wolfson Brain Imaging Centre, Addenbrookes Hospital, Cambridge or from the PET Imaging Centre, St Thomas' Hospital, London, UK. Copper-64 was prepared using a biomedical cyclotron with a nickel-64 target and supplied as $^{64}CuCl_2$(aq) in 0.1M HCl (3). An aqueous solution of copper-64 acetate, ^{64}Cu(II)-acetate, was prepared by diluting 0.2 mL of $^{64}CuCl_2$(aq) in 0.1 mol/L HCl with 0.2 mol/L sodium acetate (0.9 mL). This stock solution was used for the radiolabeling experiments. ^{64}Cu-ATSM was prepared and reformulated as previously reported (4). Radiochemical purity was confirmed by radio-TLC and radio-HPLC (>98% RCP). The pH of ^{64}Cu-acetate was adjusted to 7.0 before dilution in physiological saline.

2. In vitro and in vivo studies

In vitro cell uptake assays (% cellular associated activity)

The apparatus and protocols used for these in vitro experiments are based on those previously described (5, 6). Cell viability was determined using a Trypan blue exclusion assay pre- and post incubation under all O_2 concentrations and was found to be >94 % in both cases.

EMT6 and HT1080 cells were harvested by trypsinisation. A single cell suspension (15 mL, 1×10^6 cells mL^{-1} in DMEM supplemented with 1% (v/v) FBS and no penicillin/streptomycin) was incubated at 37°C under anoxic (95% N_2, 5% CO_2), hypoxic (75% N_2, 0.5 % O_2, 5% CO_2), or normoxic (75% N_2, 20% O_2, 5% CO_2) conditions until equilibrium was reached (30 min, as measured using an Oxford Optronics Oxylab pO_2 tissue oxygenation monitor). Cells were maintained at the respective oxygen levels throughout the assay. At various time points after the addition of 0.5-1 MBq of the desired radiolabeled compound 1 mL aliquots were removed, centrifuged to obtain the cell pellets, and the supernatant was removed. The cell-associated radioactivity and that of the supernatant were measured using a γ-counter (Hidex Triathler). The amount of ^{64}Cu activity associated with the cells (pellet) as a percentage of the total activity added was calculated. Cell-free control experiments were conducted to determine non-specific binding to centrifuge tubes.

Serum binding and stability measurements

In vitro stability studies. Samples of non-tumor-bearing CBA mouse blood were collected by heart puncture into heparin-coated vials. (The effect of the heparinised tubes on the stability of the copper complexes was investigated by radio-TLC analysis. The compounds were found to be >99% intact after 120 min incubation.) Aliquots of 500-800 μL of blood

were incubated with 0.2-0.5 MBq of the required ^{64}Cu-copper complex at 37°C for 5-120 min. To determine the amount of intact complex, samples were analysed using octanol extraction, as described below. In some cases, vials were centrifuged to obtain plasma. *In vitro* plasma incubation experiments were carried out as previously described (4, 5, 7). Briefly, 0.5 MBq of the required ^{64}Cu-labeled copper complex was incubated with 500 µL fresh mouse plasma at 37°C for 5-120 min. To determine the amount and species of protein-bound ^{64}Cu, 50 µL aliquots were withdrawn from the serum at various time-points and analysed using ethanol extraction/precipitation, as described below.

In vivo stability studies. Female, non-tumor-bearing CBA mice were injected intravenously with 1-2 MBq of the required ^{64}Cu-copper complex. 500-800 µL of blood was collected by heart puncture into heparin-coated vials at various time points after injection (n = 2 per time point). Whole blood was analysed by octanol extraction as described below. In order to analyse the species in the plasma, the vials were spun (4000 rpm, 4°C, 5 min) and the supernatant (serum) was isolated and analysed using ethanol extraction, as described below.

Protein binding and stability (ethanol extraction). Aliquots of 50 µL serum were added to 200 µL ethanol to precipitate proteins. The mixture was centrifuged until formation of a protein pellet and the supernatant was removed. The pellet was washed with 200 µL ethanol and re-centrifuged. The combined supernatants and the pellet were counted in a γ-counter to determine the percentage of protein bound activity. The supernatant was further analysed by radio-TLC (95:5 EtOAc/MeOH, where the retardation factor for ^{64}Cu-acetate = 0 and ^{64}Cu-ATSM = 0.7) or radio-HPLC to determine the amount of intact ^{64}Cu-complex present. Digital autoradiography was performed on developed radio-TLC using super resolution phosphor screens (Type SR, Perkin Elmer) and a CyclonePlus phosphor imager (PerkinElmer).

Stability in blood (octanol extraction). Octanol extraction methods were based on those previously described.(8, 9) To determine the amount of intact, octanol-extractable compound, 50 µL of whole blood was immediately added to 750 µL of n-octanol and vortexed for 1 min. The mixture was centrifuged (14,000 rpm, 5 min), the octanol phase aspirated and the octanol and pellet were counted in a γ-counter. The percentage of octanol-extractable activity, i.e. the amount of intact ^{64}Cu-ATSM was determined. The octanol phase was further analysed by radio-TLC (95:5 EtOAc/MeOH) to confirm that the extractable activity was in the form of ^{64}Cu-copper complex. Developed TLC plates were processed as above.

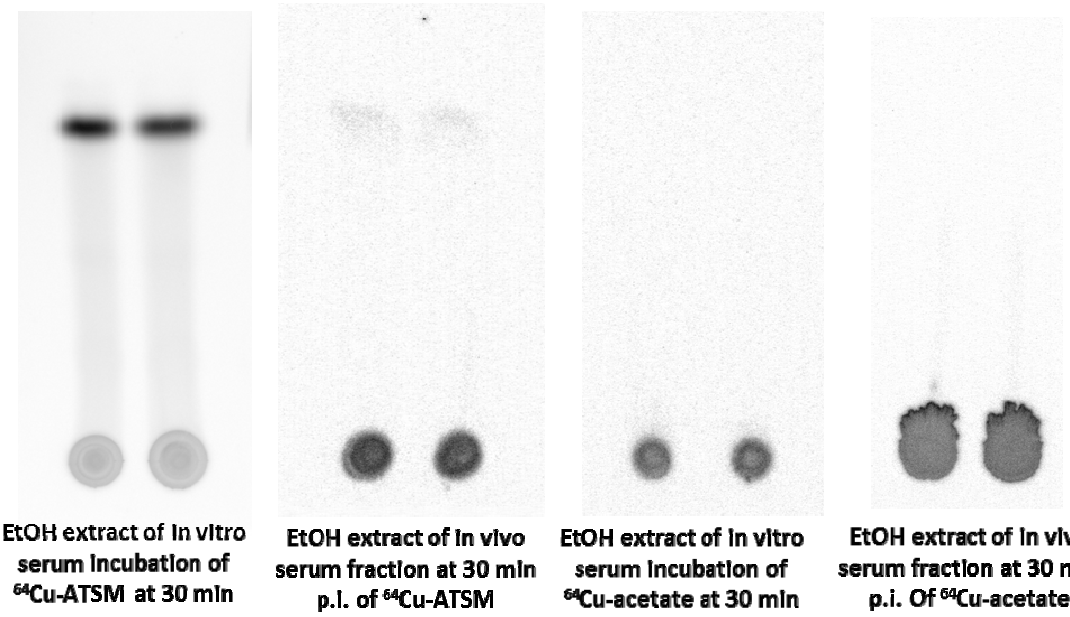
3. Correlation coefficients (Autoradiography and EF5 IHC)

The Manders' overlap coefficient is based on the Pearson's correlation coefficient with average intensity values being taken out of the mathematical expression. This new coefficient will vary from 0 to 1, the former corresponding to non-overlapping images and the latter reflecting 100% colocalisation between both images. M_1 is defined as:

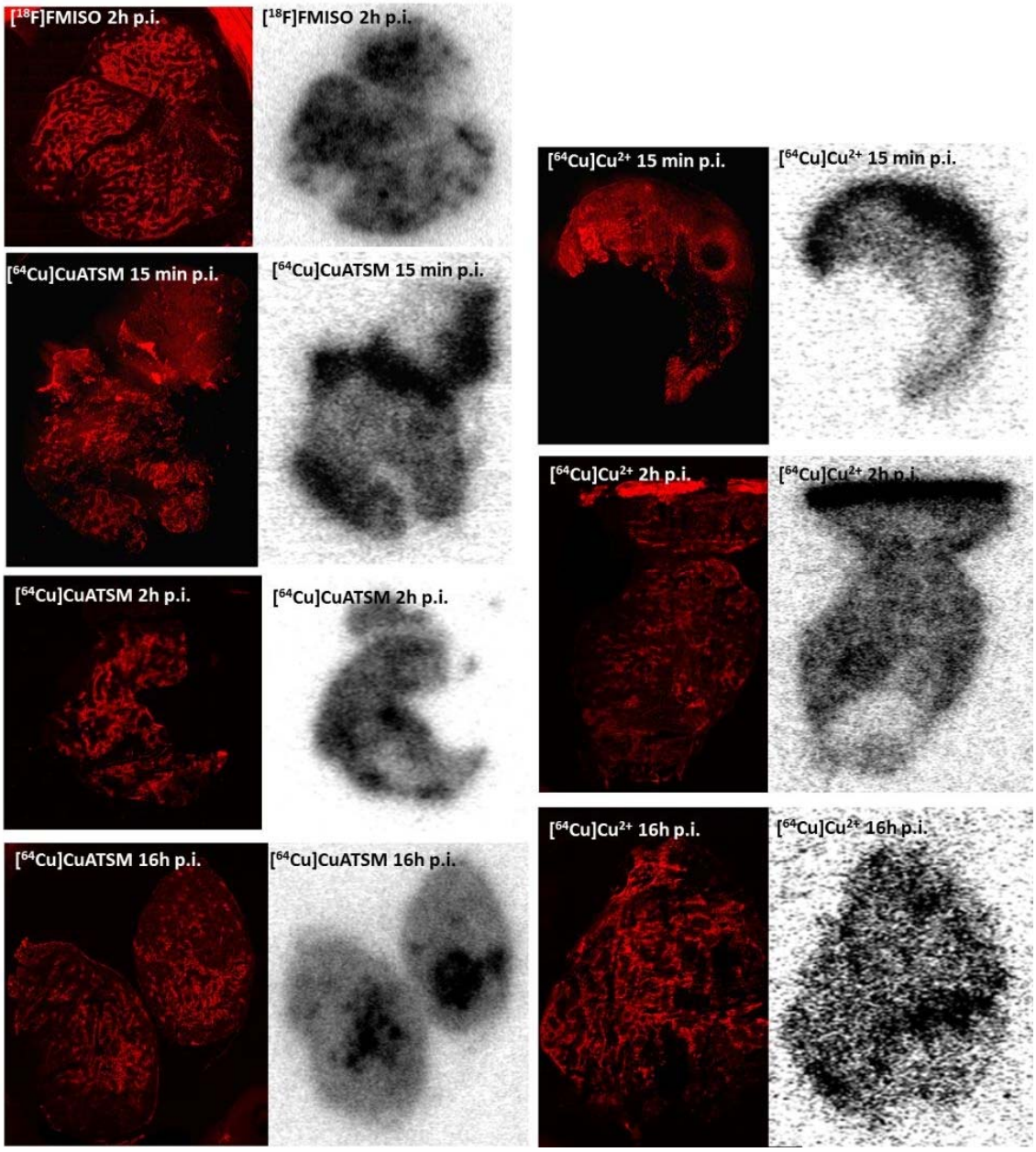
The ratio of the 'summed intensities of pixels from the autoradiography image above background for which the intensity in the EF5 image is above background' to the 'total intensity of the pixels in the autoradiography image above background'.

M_2 is defined conversely for the EF5 image. M_1 (or M_2) is a good indicator of the proportion of the autoradiography signal coincident with a signal in EF5 image over its total intensity, which even applies if the intensities in both images are really different from one another, making this method very suitable to determine correlations between different modalities. This definition makes both M1 and M2 to be perfect for colocalisation studies. To compensate for the Manders' coefficient sensitivity to noise, M_1 and M_2 may be calculated by setting a

threshold to the estimated value of background. Here, this threshold was calculated automatically using Costes' method and visually verified.(10) Costes' threshold is determined as followed: The Pearson's coefficient is calculated on the autoradiography image and the EF5 image *below* decreasing thresholds. When Pearson's correlation coefficient equals 0 (i.e. random correlation), Costes' threshold has been reached, and Manders' coefficients are determined for the image *above* the threshold.



Supplemental Figure 1. Representative radio-TLCs (95:5 EtOAc/MeOH) of ethanol phase following the ethanol extraction/precipitation method. Radio-TLCs of EtOH extract from *in vitro* incubation of ^{64}Cu-acetate and ^{64}Cu-ATSM in fresh mouse serum after 30 min incubation and radio-TLCs of EtOH extract of the serum fraction obtained at 30 min from blood of mice *injected* with ^{64}Cu-ATSM and ^{64}Cu-acetate (R_f = 0 for ^{64}Cu-acetate, R_f =0.65-0.73 for ^{64}Cu-ATSM.



Supplemental Figure 2. Representative images of ^{64}Cu or ^{18}F activity distribution and hypoxia in CaNT tumors for ^{64}Cu-ATSM, ^{64}Cu-acetate and ^{18}F-MISO, as measured by autoradiography and EF5 immunostaining at 15 min, 2 h and 16 h p.i. Autoradiography (right) and EF5 immunostaining (left) were performed on the same section. EF5 stained hypoxic areas are indicated by red, high intensity in autoradiographs is indicated by dark areas. The spatial correlation coefficients between autoradiography and EF5 staining images were obtained as outlined in Materials and Methods and the S.I.

Supplemental Table 1. Biodistribution data (%ID/g ±SD) of ^{64}Cu-ATSM (n=4), ^{64}Cu-acetate (n=3) at 120 min p.i. in female CBA mice bearing CaNT tumors that were anaesthetised with isoflurane/air for 120 min dynamic PET/SPECT imaging sessions.

%ID/g	^{64}Cu-ATSM imaging (air)	^{64}Cu-acetate imaging (air)
Blood	0.73±0.05	0.66±0.04
Tumour	1.41±0.10	1.33±0.09
Muscle	0.29±0.07	0.26±0.04
Stomach	5.97±1.49	7.11±6.26
Small intestine	9.19±1.18	5.38±0.33
Large intestine	3.92±0.47	3.59±0.87
Fat	0.45±0.06	0.36±0.09
Spleen	1.53±0.21	3.03±1.57
Liver	10.03±1.02	8.44±1.07
Kidneys	6.03±0.66	5.01±0.98
Heart	1.28±0.11	0.97±0.09
Lungs	5.49±0.56	5.28±0.62
Thyroid	-	-
Ratio T/M	5.14±1.30	5.16±0.66

Supplemental Table 2. Biodistribution data (%ID/g ±SD) for oxygen dependence of ^{64}Cu-ATSM (n=4 imaging, n=3 dissection) and ^{64}Cu-acetate (n=4 imaging, n=3 dissection) at 120 min p.i. in female CBA mice bearing CaNT tumors. Mice were either anaesthetised with 2% isoflurane/breathing gas (imaging) or awake (dissection) for 120 min biodistribution times. Mice were breathing 100% oxygen, 60% oxygen or 21 % oxygen (room air). The animals were kept at 60% or 100% O_2 for 30 min before iv injection of ^{64}Cu-acetate or ^{64}Cu-ATSM.

%ID/g	^{64}Cu-ATSM imaging (100% O_2)	^{64}Cu-ATSM dissection (100% O_2)	^{64}Cu-ATSM imaging (60% O_2)	^{64}Cu-ATSM dissection (60% O_2)	^{64}Cu-acetate imaging (100% O_2)	^{64}Cu-acetate dissection (100%O_2)	^{64}Cu-acetate imaging (60% O_2)	^{64}Cu-acetate dissection (60% O_2)
Blood	0.89±0.10	1.16±0.06	0.60±0.06	0.93±0.06	0.91±0.07	1.40±0.16	0.55±0.03	0.87±0.05
Tumour	1.10±0.10	2.24±0.01	1.11±0.07	1.42±0.11	1.46±0.10	2.24±0.25	1.15±0.04	1.34±0.09
Muscle	0.34±0.02	0.47±0.05	0.29±0.02	0.29±0.03	0.37±0.04	0.45±0.03	0.30±0.02	0.26±0.03
Stomach	6.08±1.00	5.97±0.72	3.59±0.26	2.92±0.46	1.95±0.23	10.36±1.89	5.45±0.45	3.72±0.46
Small intestine	9.87±0.53	9.92±0.22	4.15±0.47	4.37±0.52	6.52±0.74	6.47±0.85	3.35±0.46	3.06±0.77
Large intestine	3.27±0.33	5.55±0.14	2.17±0.33	2.53±0.19	3.85±0.25	7.20±1.22	2.39±0.30	4.33±0.58
Fat	0.48±0.03	0.54±.0.07	0.19±0.02	0.48±0.04	0.30±0.04	0.49±0.00	0.12±0.01	0.24±0.04
Spleen	1.70±0.09	1.63±0.14	1.01±0.11	1.69±0.11	1.33±0.12	1.85±0.13	1.51±0.15	1.70±0.23
Liver	11.71±0.77	6.86±0.84	3.55±0.43	3.03±0.11	7.60±0.85	8.20±0.65	3.98±0.48	5.13±0.80
Kidneys	5.86±0.53	5.02±0.45	2.96±0.18	5.56±0.39	6.11±0.27	5.88±0.20	3.09±0.23	4.47±0.33
Heart	1.48±0.15	2.28±0.24	0.83±0.09	1.80±0.13	1.23±0.20	2.76±0.02	0.86±0.01	1.50±0.14
Lungs	5.22±0.29	4.57±0.34	3.03±0.18	5.30±0.68	4.65±0.45	4.49±0.88	3.16±0.31	3.87±.039
Ratio T/M	3.25±0.22	4.80±0.60	3.80±0.18	4.87±0.49	3.92±0.21	4.95±0.37	3.90±0.34	5.14±0.26

Supplemental Table 3. Biodistribution data (%ID/g ±SD) of ^{64}Cu-ATSM (n=4 imaging, n=4 dissection) and ^{64}Cu-acetate (n=4 imaging, n=6 dissection) at 120 min p.i. in female CBA mice bearing EMT6 tumors that were anaesthetised with isoflurane/air (imaging) or breathing room air (dissection) for 120 min p.i.

%ID/g	^{64}Cu-ATSM imaging (air)	^{64}Cu-ATSM dissection (air)	^{64}Cu-acetate imaging (air)	^{64}Cu-acetate dissection (air)
Blood	0.52±0.05	0.77±0.06	0.58±0.03	0.89±0.09
Tumour	1.00±0.05	2.37±0.18	1.42±0.20	2.04±0.13
Muscle	0.19±0.01	0.24±0.01	0.23±0.02	0.26±0.02
Stomach	3.91±0.45	4.05±1.12	6.73±0.53	3.47±0.43
Small intestine	7.72±0.48	4.64±0.54	6.63±0.73	4.93±0.21
Large intestine	3.14±0.24	10.55±1.07	4.28±0.66	5.73±0.65
Fat	0.26±0.02	0.23±0.03	0.40±0.07	0.18±0.02
Spleen	1.38±0.05	1.32±0.16	1.18±0.15	1.60±0.21
Liver	10.40±1.36	6.63±0.29	9.73±1.32	8.46±0.55
Kidneys	5.16±0.42	4.19±0.54	6.00±1.01	5.12±0.60
Heart	1.05±0.09	1.32±0.05	1.01±0.10	1.73±0.17
Lungs	4.76±0.34	4.90±0.45	5.18±0.41	5.34±0.57
Ratio T/M	5.30±0.57	9.76±0.63	6.27±0.96	7.90±0.77

Supplemental References

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