Supplementary Information

Synthesis, complex stability and small animal PET imaging of a novel ^{64}Cu-labelled cryptand molecule

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Abbreviations

A_s	specific activity {$A_{s,eff}$ = A[GBq]/n[µmol]}
DiAmSar	3,6,10,13,16,19-hexa-aza-bicyclo-[6.6.6]eicosane-1,8-diamine
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
EDTA	ethylenediamine-*N,N,N′,N′*-tetraacetic acid
EMT-6	epithelial mammary carcinoma tumour cell line
PBS	phosphate buffered saline
NH_4OAc	ammonium acetate
NOTA	1,4,7-triazacyclononane-1,4,7-triacetic acid
rcy	radiochemical yield
TLC	thin layer chromatography

Experimental part:

General:

Compound CryptTM was synthesised as described elsewhere[1]. For all radiolabelling experiments aliquots of the following stock solutions were used:

CryptTM: 2.25 mM ethanolic stock solution

DiAmSar: purchased from *Macrocyclics*;
2.00 mM aqueous stock solution;
0.55 mM in 100 mM ammonium acetate stock solution (*99.999% trace metal basis from Sigma-Aldrich*; pH 5.5; pre-treated with Chelex-100)

NOTA: purchased from *Macrocyclics*;
25 µM, 500 µM, 2 mM, 10 mM, and 20 mM stock solutions (100 mM ammonium acetate; *99.999% trace metal basis from Sigma-Aldrich*; pH 5.5; pre-treated with Chelex-100)

EDTA: purchased from *Sigma-Aldrich*;
25 µM, 500 µM, 2 mM and 10 mM stock solutions (100 mM ammonium acetate; *99.999% trace metal basis from Sigma-Aldrich*; pH 5.5; pre-treated with Chelex-100)

^{64}Cu solution: [^{64}Cu]$CuCl_2$ in 10 mM HCl was diluted by adding 100 mM ammonium acetate buffer (*99.999% trace metal basis from Sigma-Aldrich*; pH 5.5; pre-treated with Chelex-100) yielding c_A between 2-8 MBq/µL

radio-HPLC system: Gilson HPLC system with Gilson 321 pump and UV/Vis-155; Luna® C-18 column (10 mm x 250 mm; 10 µm particle size) from *Phenomenex*; solvent A water-TFA 0.2 % v/v; solvent B acetonitrile; gradient 0-20 min 10-80% B; flow 3 mL/min; $t_{R,\,^{64}Cu\text{-}CryptTM}$ = 10.5 min.

radio-TLC system: Alugram® RP-18W from *Mackery-Nagel*; 5-10 mM EDTA solution; pH 6;
$R_{f,\,^{64}Cu\text{-}EDTA}$ = 0.8; $R_{f,\,^{64}Cu\text{-}NOTA}$ = 0.5; $R_{f,\,^{64}Cu\text{-}CryptTM}$ = 0.0; $R_{f,\,^{64}Cu\text{-}DiAmSar}$ = 0.0

Radiochemistry:

Radiosynthesis of [^{64}Cu]Cu-CryptTM:

Radiolabelling for animal studies:

To 4.0 µL (11.0 nmol) of CryptTM was added 15 µL (60-80 MBq) of [^{64}Cu]$Cu(OAc)_2$ and the mixture was shaken for 60 min at 37 °C. Radiochemical yields were determined by radio-TLC and radio-HPLC.

Optimisation of specific activity:

A) To 2 µL (5.5 nmol) of CryptTM was added 20 µL (80-100 MBq) of [^{64}Cu]$CuOAc_2$ and the mixture was shaken for 60 min at 37 °C. Radiochemical yields of > 95 % were confirmed by radio-TLC. Specific activities were determined to (16.4 ± 1.8) GBq/µmol.

B) To lower quantities of CryptTM (4.5 nmol < n_1 > 1.0 nmol; V < 5 µL) was added up to 30 µL (50-100 MBq) of [^{64}Cu]$CuOAc_2$ and the mixture was shaken for 60 min at 37 °C. Radiochemical yields in the range of 36 to 95 % were achieved. Non-CryptTM-bound [^{64}Cu]Cu^{2+} was removed by cartridge purification. To the labelling mixture was added 2 mL of de-ionised water, and the solution was transferred onto a SepPAK tC18 plus cartridge. By adding 5 mL of de-ionised water, non-bound ^{64}Cu was

eluted. Product [^{64}Cu]Cu-CryptTM was eluted with 2 mL ethanol. A maximum specific activity of 45 GBq/µmol was achieved.

[^{64}Cu]CryptTM challenge experiments:

To 4 µL ethanolic CryptTM solution (11 nmol) was added 3 µL of [^{64}Cu]Cu(OAc)$_2$ (9-15 MBq) and the mixture was shaken for 45 min at 37 °C. The yield was determined by radio-TLC. NOTA or EDTA from appropriate stock solutions (aliquots of <12 µL) in molar ratios relating to CryptTM:EDTA and CryptTM:NOTA of 100:1, 10:1, 1:1, 1:10, and 1:100 was added to the reaction mixture. The mixtures were continuously shaken at 37 °C. Radio-TLCs of each mixture were developed after 5 min, 10 min, 20 min, 40 min, 100 min, and 18 h.

LogP determination of [^{64}Cu]Cu-CryptTM:

[^{64}Cu]Cu-CryptTM was prepared as described above (rcy of 100 %). Three different octanol-PBS mixtures were prepared. Therefore, 5 µL aliquots were added to 495 µL PBS (pH 7.4). After adding 500 µL octanol, the two-phase system was shaken at room temperature for 5 min and subsequently centrifuged at 2000 rpm for 3 min. Three times 100 µL of each fraction was taken for measurement of radioactivity. The log P value was estimated with log P = -0.3.

Radiosynthesis of [^{64}Cu]Cu-Diamsar:

[^{64}Cu]Diamsar was prepared as described for [^{64}Cu]Cu-CryptTM (rcy of 100 %). Radiolabelling was already completed after 10 min.

[^{64}Cu]Cu-Diamsar challenge experiments:

To 183 µL of 0.55 mM Diamsar solution was added 2 µL of [^{64}Cu]Cu(OAc)$_2$ solution and shaken for 30 min at 37 °C achieving rcy of 100 %. This solution was used for challenge experiments. For a molar ratio of 1:1, 36.7 µL of [^{64}Cu]Cu-Diamsar was added to 10 µL of 2 mM NOTA stock solution or 2 mM EDTA stock solution. For a molar ratio of 1:100, (Diamsar to EDTA or NOTA) 5.0 µL of [^{64}Cu]Cu-Diamsar was added to 27.2 µL of 10 mM NOTA stock solution or 10 mM EDTA stock solution. Mixtures were shaken at 37 °C and samples were analysed by radio-TLC after 10 min, 30 min, 60 min, and 120 min.

LogP determination of [^{64}Cu]Cu-DiAmSar:

Three different octanol-PBS mixtures were prepared. Therefore, aliquots of 5 µL was added to 495 µL PBS (pH 7.4). After adding 500 µL of octanol the two-phase system was shaken at room temperature for 5 min and subsequently centrifuged at 2000 rpm for 3 min. Three times 100 µL of each fraction was taken for measurement of radioactivity. The log P value was estimated with log P = -3.8.

Radiopharmacological studies:

Distribution of [^{64}Cu]CryptTM in blood and metabolic profiling:

Typically, 5 MBq of [^{64}Cu]Cu-CryptTM in 100-150 µL of saline (0.9%) was injected as a bolus through a catheter into the tail vein of isoflurane anesthetised BALB/c mice bearing EMT-6 tumours. Before radiotracer injection, mice were heparinised by subcutaneous injection of 50 µL heparin (1000 I. U.) and kept under anaesthesia during the course of the experiment. At 60 min p.i., the animal was sacrificed and a whole blood sample (approximately 500 µL) and urine were collected. Blood cells were separated by immediate centrifugation (5 min at 13,000 rpm). Proteins within the sample were precipitated by adding methanol (ca. 800 µL) to the supernatant following a second centrifugation step (5 min at 13,000 rpm). The activity present in each fraction was measured using the gamma-counter.

Small animal PET imaging in BALB/c mice bearing EMT-6 tumours using [^{64}Cu]Cu-CryptTM and [^{64}Cu]Cu-DiAmSar:

All animal experiments were carried out in accordance with guidelines of the Canadian Council on Animal Care (CCAC) and were approved by the local animal care committee of the Cross Cancer Institute. Positron emission tomography (PET) experiments were performed using BALB/c mice bearing EMT-6 tumours. The mice were not fasted prior to imaging experiments. The animals were anesthetised through inhalation of isoflurane in 40% oxygen/60% nitrogen (gas flow, 1 L min^{-1}) and body temperature was kept constant at 37°C for the entire experiment. Mice were positioned and immobilised in the prone position with their medial axis parallel to the axial axis of the scanner and their thorax, abdomen and hind legs (organs of interest: heart, kidneys, bladder, liver) in the centre of the field of view of the microPET® R4 scanner (Siemens Preclinical Solutions, Knoxville, TN, USA). A transmission scan for attenuation correction was not acquired. 4-6 MBq of either [^{64}Cu]Cu-CryptTM and [^{64}Cu]Cu-DiAmSar in 100-150 µL saline (0.9%) was injected through a needle catheter into the tail vein. Data acquisition continued for 60 min in 3D list mode. The frames were reconstructed using MAP. The pixel size was 0.085 cm by 0.085 cm by 0.12 cm and the resolution in the centre field of view was 1.8 mm. No correction for partial volume effects was performed. The image files were further processed using the ROVER v 2.0.21 software (ABX GmbH, Radeberg, Germany). Masks for defining 3D regions of interest (ROI) were set and the ROIs were defined by thresholding. ROI time-activity curves (TACs) were generated for subsequent data analysis. Standardised uptake values (SUV = (activity/mL tissue)/(injected activity/body weight), mLg^{-1}) were calculated for each ROI. Data are expressed as means ± SEM from n investigated animals. All TACs were constructed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA).

References:

1. J. C. Knight, R. Prabaharan, B. D. Ward, A. J. Amoroso, P. G. Edwards, and B. M. Kariuki, *Dalton Trans.*, 2010, **39**, 10031–3.