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Supplemental Information

A PET Tracer For Renal Organic Cation Transporters, ^{11}C-metformin: Radiosynthesis and Preclinical Proof-of-Concept Studies

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Target Organ	Dosis (mSv/MBq)	
	Baseline	**Metformin**
Adrenals	3.52E-03	3.17E-03
Brain	1.49E-03	1.34E-03
Breasts	1.66E-03	1.51E-03
Gallbladder Wall	4.47E-03	4.16E-03
LLI Wall	3.09E-03	3.45E-03
Small Intestine	2.79E-03	2.76E-03
Stomach Wall	2.45E-03	2.24E-03
ULI Wall	2.83E-03	2.75E-03
Heart Wall	3.54E-03	3.34E-03
Kidneys	2.77E-02	2.09E-02
Liver	1.88E-02	1.80E-02
Lungs	2.20E-03	2.01E-03
Muscle	2.17E-03	2.13E-03
Ovaries	3.12E-03	3.42E-03
Pancreas	3.27E-03	2.97E-03
Red Marrow	2.05E-03	1.95E-03
Osteogenic Cells	2.73E-03	2.52E-03
Skin	1.59E-03	1.49E-03
Spleen	2.49E-03	2.20E-03
Testes	2.37E-03	2.58E-03
Thymus	1.88E-03	1.71E-03
Thyroid	1.71E-03	1.54E-03
Urinary Bladder Wall	5.64E-02	8.28E-02
Uterus	4.53E-03	5.53E-03

Total Body	2.72E-03	2.62E-03
Effective Dose (mSv/MBq)	**6.04E-03**	**7.30E-03**

Supplemental Table 1 Biodistribution and organ dosimetry in a pig after ^{11}C-metformin administration at baseline condition and during metformin infusion.

Supplemental Materials and Methods

Chemicals

Ammonium dihydrogenphosphate, phosphoric acid, metformin hydrochloride, cimetidine, quinine, cisplatin, tetrabutylammonium hydrogen phosphate and acetone were obtained from Sigma-Aldrich Ltd. and used as received. 1-methylbiguanide (as hydrochloride salt) was from LGC Standards. Water (sterile) and aqueous NaOH (3 M; sterile) were prepared by the pharmacy at Aarhus University Hospital.

Radiochemistry

Cyclotron-produced $^{11}CO_2$ or $^{11}CH_4$ was converted to $^{11}CH_3I$ and further to $^{11}CH_3OTf$ and then directed to the reaction vial containing the precursor in acetone and sodium hydroxide as a supporting base. After heating the reaction mixture for 5 min at 70 degrees, the crude product is diluted with 1 ml water and purified by reverse phase HPLC on a Luna SCX (10 μm, 10x250 mm) (Phenomenex) column with aqueous $(NH_4)_2HPO_4$ (100 mM) as mobile phase (flow 15 ml/min; $\lambda = 232$ nm). The fraction from the chromatographic separation corresponding to 11C-metformin was collected (from ca. 6.5 - 8 min) and passed over a sterile filter (Cathivex-GS 0,22 μm (Millipore)) into the final sterile product vial.

The radiochemical purity of the synthesized 11C-metformin was determined by analytical HPLC using an Ultimate® 3000 system (Dionex) (λ= 232 nm) connected to a GabiStar radio-detector (Nuclear Interface). The chromatographic column was a Luna 5µ SCX 100A (150 x 4.6 mm) (Phenomenex) with aqueous $(NH_4)_2HPO_4$ (100 mM; adjusted to pH 5 with H_3PO_4) as eluent (isocratic, 2.5 mL/min). The chromatographic data were analyzed using Chromeleon software (Dionex) (version 6.80). The identity of 11C-metformin (retention time: 3.4 min) was confirmed by co-injection of reference material. Additional chromatographic conditions were used for the identification of ^{11}C-metformin: Luna C18(2) 150x4.6 mm (Phenomenex) eluted with 30% acetonitrile 70% 10 mM sodium octanesulfonate (pH adjusted to 3 with sulfuric acid). On this HPLC system, 11C-metformin had a retention time of 5 min.

Supplemental Experimental Procedures

Animal studies

The studies were performed according to the Danish Animal Experimentation Act and the European convention for the protection of vertebrate animals used for experimental and other purposes (ETS No. 123). The animal protocols were approved by the board of the Institute of Clinical

Medicine at Aarhus University according to the license for the use of experimental animals issued by the Danish Ministry of Food.

Autoradiography studies

Lewis rats (250 – 285 gram, male) were injected intravenously in a tail vein with 10-20 MBq ^{11}C-metformin under isoflurane anaesthesia. Two minutes after tracer injection rats were killed by decapitation and kidney and liver tissue were rapidly removed, cooled to -40 degrees in isopentane. Ten micron thick slices were cut, shortly dried and transferred to a metal cassette and exposed to a phosphor imaging plate for 1 hour. Subsequently the plates were read using a FujiBAS ImageReader.

MicroPET experiments in rats

Female Sprague Dawley rats (240 – 310 gram) were microPET scanned (microPET R4; CTI Concorde, Knoxville, TN, USA) for 90 min with dynamic recording under isoflurane anesthesia after tail vein injection of between 2 and 8 MBq/100 g body weight ^{11}C-metformin (specific activity 30 – 90 GBq/micromole, 0.01 – 0.1 microgram metformin). Likewise, microPET experiments were performed after iv pretreatment with Metformin (50 mg/kg, 1 min before tracer administration), Cimetidine (30 mg, 15 min before tracer administration), Quinine (2 mg, 2 min before

tracer administration), Tetrabutylammonium hydrogensulphate (TBA, 3 mg/kg, 5 min before tracer administration). Preparation and handling of the rats during scans were as previously reported (*37*). For all rats anesthesia was induced in a chamber filled with 5% isoflurane in a mixture of oxygen (O_2) (0.4 L/min) and air (1.5 L/min). After the induction of anesthesia, the head of the animal was positioned in a custom-built Plexiglas head holder and the anesthesia maintained with a cone mask delivering isoflurane (1.8–2.0%) in O_2(0.4 L/min) and air (1.5 L/min) fitted to the head holder. A catheter was inserted transcutaneous into the tail vein for injection of ^{11}C-metformin and/or drugs. During the experiments rectal temperature was maintained close to 36.5°C with a heat lamp regulated by a thermostat. Before each PET scan, we obtained a 10-min attenuation scan with a ^{68}Ge point source. Dynamic emission recordings were initiated upon injection of a dose of ^{11}C-Metformin, between 2 and 8 MBq/100 g body weight, followed by a 90 -120 min long emission recording of 37 - 40 frames increasing in duration from 5 s to 10 min. After the PET sessions, animals were decapitated. Attenuation-corrected dynamic emission images were reconstructed by 3D-filtered back projection resulting in a 128 × 128 × 63 matrix.

PET/CT examination of pigs

Six pigs (3-months old female Danish Landrace and Yorkshire cross-breed; body weight 35–41 kg) were fasted for 18 h with free access to water. The animal was premedicated with midazolam and s-ketamine, anaesthetized with a mixture of midazolam, s-ketamine and propofol, and mechanically ventilated as previously described (*38*). Catheters (Cordis) were inserted into the femoral vein and artery for intravenous administrations and blood sampling, respectively (*39*). The animal was placed on a thermostatically-controlled heating blanket, keeping the rectal temperature 38.5-39.5 ^{o}C. By adjusting the mechanical respiration, arterial blood pCO_2, pO_2, and pH were kept between 5.3-7.2 kPa, 12-25 kPa, and 7.35-7.45, respectively. Blood glucose level was kept between 5.0-6.7 mM. After completion of the experiment, the animal was euthanized with an overdose of pentobarbital (100 mg/kg).

For PET/CT studies, the pigs were placed supine in a Siemens Biograph 64 Truepoint PET/CT scanner with a 21.6 cm transaxial PET field-of-view. A CT scan (250 effective mAs with CAREDose4D, 120 kV, pitch 1.0, slice thickness 2.0 mm) was performed for definition of anatomical structures and attenuation correction of the PET recordings. PET scanning was performed as described by Alstrup & Winterdahl(*40*).

In pig no. 1, ^{11}C-Metformin administration was given to determine the biodistribution of the tracer by means of whole-body PET/CT recordings. A bolus+constant infusion with metformin (10 mg/kg + 0.1 mg/kg/min; to mimic plasma concentration of metformin in the clinical setting) was given 20 min prior to a second ^{11}C-metformin administration with concomitant whole-body PET/CT recordings.

Pigs no. 2, 3 and 4 underwent either 90 or 120-min dynamic PET/CT recordings (list-mode) with kidneys and liver in field-of-view using a single 10-sec long intravenous administration of 48 - 295 MBq ^{11}C-metformin. In Pig 4, a second dynamic ^{11}C-metformin PET/CT was preceded by intravenous 20-second infusion of 1.5 g metformin to investigate possible inhibition of the transport of ^{11}C-metformin.

The decay-corrected dynamic PET data were reconstructed using the iterative TrueX algorithm (3 iterations, 21 subsets) and post-filtered using a 3 mm Gaussian filter yielding 3D-images of 168 x 168 x 109 voxels. Time-activity curves (TACs) were generated from volumes of interest (VOIs) drawn in renal cortex, renal medulla and liver tissue using fused PET/CT images.

To estimate the pharmacokinetics of ^{11}C-metformin in the renal cortex we used a simple 1-tissue compartment model with K_1 as rate constant for the transport from plasma into tissue and k2 as tissue clearance parameter. The PET-derived distribution volume was calculated as the ratio K_1/k_2. Alternatively, the distribution volume in renal cortex could be estimated as the ratio of the area-under-the-curve (AUC) for renal cortex to that of plasma, each calculated at presumed steady-state 60 min post-injection. From the CT scan, we derived the renal cortex volume V_{cortex}.

Urine and renal clearance of ^{11}C-metformin and ^{51}Cr-EDTA

Pigs no. 5 and 6 were used to evaluate the urine clearance of ^{11}C-metformin and for comparison with the glomerular filtration rate (GFR) measured with ^{51}Cr-EDTA using a constant infusion clearance technique (*41*). Two hours before the start of the PET/CT scans, the pig received a bolus (2 MBq in 10 mL) + constant infusion (1 MBq in 5 mL per hour) of ^{51}Cr-EDTA. Dynamic 120-min PET recordings for both pigs were initiated shortly before administration of ^{11}C-metformin which was injected as a bolus (14 MBq) followed immediately with constant infusion of 94 MBq per hour. In pig 5, the PET scan was repeated with 11 MBq as bolus plus 91 MBq/hour. Blood samples of 2 ml were drawn every 10 min from a femoral artery. Urine was

collected quantitatively for four 30-min periods (0-30, 30-60, 60-90 and 90-120 min) Plasma and whole blood samples of 0.5 ml were counted in a well-crystal scintillation detector, together with plasma and urine blanks, and a dilution of the urine, respectively. All tubes were counted twice, first for ^{11}C-metformin and 6 hours later for ^{51}Cr-EDTA.

Urine clearance = U x V / P

Where U is the activity excreted in the urine collection time interval, V is the urine volume and P is the activity in the plasma sample.

Biodistribution, metabolites and protein binding

To examine the arterial input of ^{11}C-metformin blood was sampled (0.5 mL) from the femoral artery at 12 x 5, 6 x 10, 6 x 30, 5 x 60, and 8 x 600 seconds and radioactivity concentrations were measured in a well counter (Packard Biosciences) that was cross-calibrated to the PET camera. For all pigs, additional blood samples were collected 2, 5, 10, 20, 40, 60, and 90 min after tracer administration for determination of ^{11}C-metabolites in plasma.

From Pigs 1, 5 and 6 urine samples were collected 15, 30, and 90 min after administration of ^{11}C-metformin to allow for analysis of possible radioactive metabolites.

The plasma and urine samples were fractionated by HPLC (monitored by serial UV detection (lambda = 232 nm) and radiodetection) and radioactivity concentrations were measured in the well counter. HPLC conditions was LUNA™ SCX (Phenomenex®, 250 × 10 mm) using 100 mM $(NH_4)_2HPO_4$, pH 5, as eluent delivered at 5 ml/min.

Plasma protein binding of ^{11}C-metformin was determined by the ultrafiltration method (*42*) at baseline (pigs 1, 5 and 6) and post metformin administration (pig 1).

The biodistribution study was performed twice, 120 min apart (6 times the radioactive half-time of 20.4 min), first at baseline conditions (153 MBq) and then during infusion of unlabelled metformin (175 MBq). ^{11}C-metformin was administrated intravenously, followed by six successive whole-body PET recordings (with six bed-positions) with a progressive increase in scan duration per bed position: 1, 1.5, 2, 3, 4 and 5 min, respectively. Organs with high accumulation of tracer relative to

surrounding tissue were identified by visual inspection (kidney, liver, heart wall and urinary bladder contents) and were included as individual source organs. The salivary glands could be identified, but their contribution was small and could be ignored in the calculations. The total liver radioactivity was estimated as the radioactivity concentration in a liver VOI multiplied by the liver volume. In the similar way, the total radioactivity was estimated in kidney and heart wall. The urinary bladder contents were measured using urine samples. For each source organ, the time course of the non-decay-corrected total radioactivity was generated. Data were extrapolated from pig (40 kg) to human (74 kg) and recalculated into time courses of fractions of injected dose (% ID). Residence times were computed as the trapezoidal sum of the time course of % ID assuming that the radioactivity decayed only by physical decay after the last scan. Residence time for the rest of the body was calculated as the total body residence time minus the sum of the residence times from the source organs. The residence times were entered into OLINDA/EXM 1.0 (*43*) to compute absorbed doses using the male reference phantom and to obtain effective dose values according to ICRP 60 (*44*).

In vitro **studies**

LLC-PK_1 is a porcine kidney proximal tubule cell line (*45*), which is widely used for cellular studies on kidney epithelial transport. LLC-PK_1 cells were grown in high-glucose D-MEM supplemented with pyruvate, non-essential amino acids and Hepes buffer. Before experiments, LCC-PK_1 cells were seeded in 5 cm plastic Petri dishes and grown until near confluent. The day prior to experiment, media was changed and cells were placed on an orbital shaker overnight in a cell incubator (in order to prevent pericellular oxygen/nutrient depletion in the dense cell cultures). Fifteen min prior to the addition of tracer, cells were treated with vehicle (saline) or 2.5 mM or 10 mM metformin. Subsequently, 1-2 MBq ^{11}C-metformin was added to each dish, and cells and media samples were sampled 5, 10, 15 and 30 min after tracer addition. In short, cells were washed three times in saline, scraped from the bottom and transferred to counting vials and radioactivity was measured with a Packard Well counter. For each dish, radioactivity was normalized for small differences in tracer concentration as measured in medium samples. Other petri dishes were trypsinized and cells were counted using a hemacytometer and ^{11}C-metformin retention was finally expressed as percentage of total added dose per 10^6 cells. In another experiment, tracer clearance kinetics was assessed. In short, cells were loaded with tracer for 30 min (as above), washed thoroughly in saline and then either collected and analyzed for radioactivity as above, or reincubated

in tracer free medium and collected at various time points. Clearance was expressed as remaining fraction relative to that measured immediately following the 30 min tracer-loading period.

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