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Synthesis and evaluation of a ^{11}C-labelled angiotensin II AT_2 receptor ligand

Ola Åberg,[a‡] Marc Stevens,[b] Jonas Lindh,[b] Charlotta Wallinder,[b] Håkan Hall,[c] Azita Monazzam,[c] Mats Larhed,[b*] and Bengt Långström,[a,d†]

Three ^{11}C-radiolabelled high-affinity nonpeptide AT_2 receptor-selective ligands were synthesized and one of these was evaluated as positron emission tomography (PET) tracer. The labelling reaction was performed via palladium(0)-mediated aminocarbonylation of the aryl iodide substrate using [^{11}C]carbon monoxide as the labelled precursor. As an example, starting with 10.0 GBq [^{11}C]carbon monoxide, 1.10 GBq of the product *N*-butoxycarbonyl-3-[4-(*N*-benzyl-[^{11}C]carbamoyl)-phenyl]-5-isobutylthiophene-2-sulphonamide [^{11}C]4d was obtained in 36% decay-corrected radiochemical yield (from [^{11}C]carbon monoxide), 42 min from end of bombardment with a specific activity of 110 GBq · μmol^{-1}. The *N*-isopropyl-[^{11}C]carbamoyl-analogue [^{11}C]4c (radiochemical purity >95%) was studied employing autoradiography, organ distribution, and small animal PET. *In vitro* autoradiography showed specific binding in the pancreas and kidney. Organ distribution in six rats revealed a high uptake in the liver, intestine, kidney, and adrenals. Small animal PET showed rapid and reversible uptake in the kidneys followed by accumulation in the urinary bladder suggesting fast renal excretion of the tracer. In addition, high accumulation was also seen in the liver. For future studies, more metabolically stable tracers will need to be developed. To the best of our knowledge, this is the first attempt of the use of PET imaging for the detection of expressed, fully functional AT_2 receptors in living subjects.

Keywords: Carbon-11; ^{11}C; [^{11}C]carbon monoxide; carbonylation; angiotensin II; AT_2; PET; autoradiography

Introduction

The renin–angiotensin system (RAS) plays an important role in the regulation of blood pressure and the octapeptide angiotensin II (Ang II) is the effector peptide. Ang II mediates its effects via the two receptors AT_1 and AT_2. The AT_1 receptor is well known and is responsible for the negative effects associated with an overactivation of the RAS, such as vasoconstriction, salt- and water-retention, and hypertrophy. The effects mediated by the AT_2 receptor are not yet fully understood but many of them oppose the effects mediated by the AT_1 receptor, for example vasodilatation and anti-proliferation.[1] The AT_2 receptor is mainly expressed in foetal tissues and expression drops rapidly after birth. In the healthy adult, expression is concentrated to adrenal glands, uterus, ovary, vascular endothelium, heart, and distinct areas of the brain.[2] During pathological conditions such as myocardial infarction,[3] brain ischaemia,[4] renal failure,[5] and Alzheimer's disease upregulation of the AT_2 receptor has been reported.[6] The distribution of AT_2 receptors has, however, been investigated *in vitro* not only with ^{125}I labelled AT_2-selective[7] and non-selective ligands but also by immunological methods or by the detection of mRNA. Clearly, there is a need for a method, which allows the observation of functional AT_2 receptors in living species, under healthy as well as pathological conditions.

Positron emission tomography (PET) can be employed to visualize receptor and enzyme concentrations in biological systems, and its use is gaining in popularity in drug development.[8] Only a very small amount of radiolabelled chemical entity is necessary for biological experiments due to the high specific activity afforded by the radionuclide used, in this study ^{11}C, which decays by positron emission (β^+, $t_{1/2}$ = 20.4 min). Therefore, the toxicity risk associated with pharmacological doses is greatly reduced, decreasing the timespan from lead compound to first tests in man in accordance with the PET microdosing concept.[9] This can be contrasted to the tedious and costly toxicology studies required when pharmacologically active doses are administered.

[a]*Department of Biochemistry and Organic Chemistry, BMC, Uppsala University, Box 576, SE-751 23, Uppsala, Sweden*

[b]*Department of Organic Pharmaceutical Chemistry, BMC, Uppsala University, Box 574, SE-751 23, Uppsala, Sweden*

[c]*Uppsala ASL, GE Healthcare, Box 967, SE-751 09, Uppsala, Sweden*

[d]*Uppsala Imanet, GE Healthcare, Box 967, SE-751 09, Uppsala, Sweden*

**Correspondence to: Mats Larhed, Department of Organic Pharmaceutical Chemistry, BMC, Uppsala University, Box 574, SE-751 23, Uppsala, Sweden. E-mail: Mats.Larhed@orgfarm.uu.se*

†*Correspondence to: Bengt Långström, Department of Biochemistry and Organic Chemistry, Uppsala University, Box 967, Uppsala, Sweden 75109. E-mail: bengt.langstrom@biorg.uu.se*

‡*Current address: Imperial College, Hammersmith Campus, Faculty of Medicine, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK.*

Recently several classes of potent, selective, and druglike AT_2 receptor agonists have been reported.[10,11] One of these, the imidazole structure M024 (Figure 1), has been utilized as a research tool[12] and shown to improve heart function after myocardial infarction in rats.[13] By introducing a positron-emitting radionuclide in any of these ligand classes, selective AT_2 receptor PET tracers could be synthesized. An optimal tracer would enable tissue distribution and receptor density studies of the AT_2 receptor in both healthy and pathological tissues. Among these ligand classes, we concluded the benzamides[11] to be the most suitable for the introduction of a ^{11}C radionuclide, and the most feasible labelling position is the amide carbonyl. Such radiolabelling can be carried out in a facile manner using a transition-metal-mediated aminocarbonylation.[14] A three-component system consisting of a substrate, [^{11}C]carbon monoxide, and a nucleophile is also ideal for the synthesis of compound libraries. This concept has been used on a laboratory scale employing microwave techniques and *in situ* produced carbon monoxide.[15] In small-scale labelling chemistry, the use of [^{11}C]carbon monoxide and a pressurized reactor system has enabled labelling of a whole array of functional groups.[16] This technique has been used to synthesize libraries of potential PET tracers.[17–19] Furthermore, we recently reported the radiolabelling of the selective AT_1 receptor ligand eprosartan using [^{11}C]carbon monoxide as a potential imaging agent for *in vivo* characterization of aldosterone-producing adenomas.[20]

The alternative Grignard method utilizing [^{11}C]carbon dioxide has been used to synthesize amides via the corresponding [1-^{11}C]acid chlorides.[21] Grignard reagents have also been used in the direct synthesis of ^{11}C-labelled amides from [^{11}C]carbon dioxide.[22,23] A drawback of these methods is that Grignard reagents are very moisture-sensitive and do not tolerate acidic functional groups such as the sulphonyl carbamate moiety present in the scaffold of the AT_2 agonists. In addition, Grignard reagents are prone to react with atmospheric carbon dioxide, which leads to isotopic dilution and lower specific activity. The use of [^{11}C]carbon monoxide generally affords higher specific activity due to the low abundance of carbon monoxide in the atmosphere.

We herein report carbonylative ^{11}C-radiolabelling of three selected high-affinity AT_2 receptor-selective ligands. We also report the biological evaluation of one of the labelled compounds using frozen tissue autoradiography, organ distribution in rat, and small animal PET.

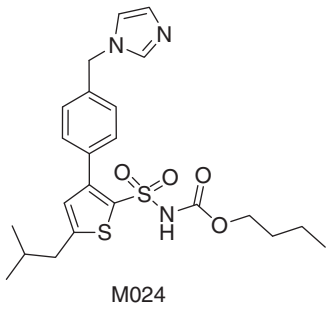
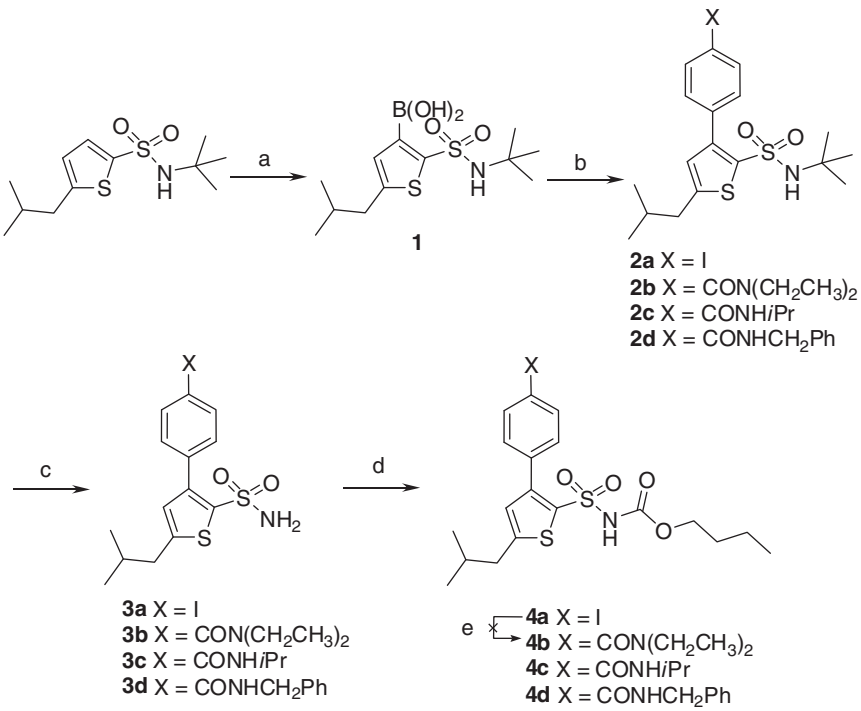


Figure 1. The first selective nonpeptide AT_2 receptor agonist.

Results and discussion

Synthesis of precursors and references

The synthesis of labelling precursors **3a** and **4a**, as well as reference substances **3b** and **4b–d**, was performed as outlined in Scheme 1. The preparative route starts from *N*-(*tert*-butyl)-5-isobutylthiophene-2-sulphonamide synthesized using methodology described by Kevin *et al.*, which was then treated with



Scheme 1. (a) (i) *n*-BuLi in THF. (ii) $B(OiPr)_3$. (iii) 2 M HCl. (b) *p*-Substituted aryl iodides, $Pd(PPh_3)_4$, Na_2CO_3, H_2O, DME, EtOH. (c) BCl_3, CH_2Cl_2. (d) *n*-Butyl chloroformate, Et_3N, CH_2Cl_2. (e) $Pd(OAc)_2$, DBU, $Mo(CO)_6$, diethylamine, THF.

n-BuLi and triisopropylborate, yielding thiophene **1**.[24] The heteroaryl boronic acid **1** was then coupled to different *p*-substituted aryl iodides in a microwave-assisted Suzuki-reaction,[25] using Na_2CO_3 as the base and *tetrakis*(triphenylphosphine)palladium(0) [$Pd(PPh_3)_4$] as the catalyst. The coupling reaction of **1** with *p*-diiodobenzene to was noticeably cleaner at 110°C than at 120°C, affording a quantitative yield of **2a**. Compounds **2a–d** were deprotected using BCl_3 to yield the free sulphonamide **3a-d**.[26] The sulphonamides were either carefully purified for later use in labelling experiments or directly acylated using *n*-butyl chloroformate and TEA (triethylamine) to yield the final products **4a–d**.[11] An alternative synthetic route to **4b** was initially tried starting from **4a** using palladium(II)-acetate [$Pd(OAc)_2$], DBU (1,8-diazabicyclo[5.4.0]undec-7-ene), molybdenum hexacarbonyl [$Mo(CO_6)$], Fu's salt ([(*t*-Bu)$_3$PH]BF_4), and diethylamine in THF, but resulted in a complex mixture of products and was abandoned.

Labelling chemistry

The radiolabelling was performed as outlined in Scheme 2. The reaction is believed to proceed via metal-coordination of [^{11}C]carbon monoxide to the palladium–aryliodide complex followed by a 1,1-insertion to form the palladium–[^{11}C]acyl complex, and then to give the desired amide product by nucleophilic attack by the amine.[14] As a consequence of the small amount (10–100 nmol), low partial pressure (in the order of 8–80 Pa) and short half-life of [^{11}C]carbon monoxide, all other reagents were used in stoichiometric excess to promote as high [^{11}C]O-conversion as possible during the 5-min reaction time. Repetitive turns in the catalytic cycle of the palladium complex were not likely to occur because of the large excess of palladium and thus the reaction is referred to as metal-mediated rather than metal-catalysed (Scheme 2). [^{11}C]O, $Pd(PPh_3)_4$, amine nucleophile, THF, 110°C, 5 min.

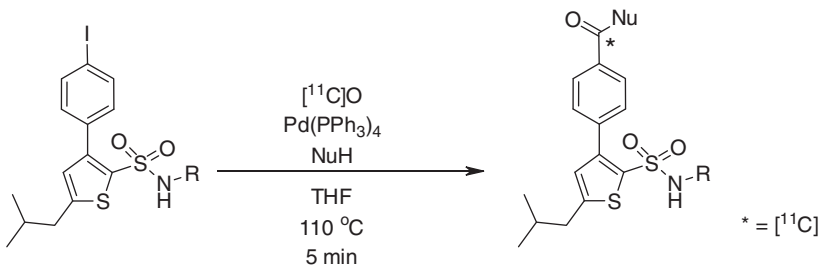
Appropriate concentrations of the reactants and reagents are of the utmost importance for high yields and subsequent purification. In previous aminocarbonylations using [^{11}C]carbon monoxide, the aryl halides were successfully used in 2–5 equiv excess to the palladium complex in order to facilitate the oxidative addition, but when excess of sulphonyl carbamate **4a** was used low radiochemical yields were obtained (Table 1, Entries 2 and 10).[14] In a recent study, it was shown that sulphonamides may serve as nucleophiles in a carbonylation reaction to give acyl sulphonamides.[27] Thus, employing an excess of an aryl halide possessing a nucleophilic functionality, such as a sulphonamide (**3a**) or a sulphoncarbamate (**4a**), might result in an undesired competition between the external amine and the amide nucleophile in the carbonylation reaction. When aryl halide **4a** was used as a substrate, a low concentration of amine generally gave poor yields (Table 1, Entries 1, 2, and 10), while increasing the concentration of amine improved the outcome up to a certain limit (500 mM, Table 1, Entries 3 and 4).

The observation of a more hydrophilic-labelled compound as the major by-product in many experiments led us to suspect that the *n*-butyl carbamate moiety was not stable during the labelling reaction. However, the by-product did not co-elute with the suspected hydrolysis product **3b** on analytical HPLC. In an attempt to avoid the unidentified by-product, we briefly explored the possibility of a two-step route to [^{11}C]**4b** starting with aryl-iodide **3a**. In contrast to observations using **4a** as substrate, no significant increase in radiochemical yield was observed when **3a** was used with high concentration of amine (Table 1, Entries 11 and 12). In both cases, the main part of the radioactivity was instead bound to very lipophilic compounds, possibly products arising from the sulphonamide itself acting as the nucleophile. This by-product could also be the palladium–[^{11}C]acyl complex, an intermediate which has been previously observed in related carbonylation reactions.[17] However, subsequent additional addition of diethylamine to a final concentration of 1.5 M and heating at 70°C for 20 min did not increase the yield of the desired product in this case (Table 1, Entry 11). All conditions tested gave poor yields of [^{11}C]**3b**; thus, the two-step route was abandoned.

In summary, the best conditions found were a 1:1 ratio of aryl halide **4a**:palladium complex, temperatures between 90 and 120°C, and high concentration of amine, which gave comparable yields 38–44% (Table 1, Entries 6–8).

Synthesis of labelled analogues

Compound libraries and combinatorial chemistry have since the early nineties been extensively used for acceleration of SAR-analyses and lead optimization processes in drug discovery.[28] The development of a micro-autoclave synthesis system for handling [^{11}C]carbon monoxide has made it possible to synthesize libraries of compounds labelled with ^{11}C in a combinatorial fashion as recently shown for labelled analogues around the lead compounds WAY-100635,[17] DAA-1106[19], a dual PDGFRβ/ VEGFR-2 inhibitor[18] and an irreversible EGFR



3a R = H
4a R = COOnBu

[^{11}C]**3b** NuH = Diethylamine, R = H
[^{11}C]**4b** NuH = Diethylamine, R = COOnBu
[^{11}C]**4c** NuH = Isopropylamine, R = COOnBu
[^{11}C]**4d** NuH = Benzylamine, R = COOnBu

Scheme 2. [^{11}C]O. $Pd(PPh_3)_4$, amine nucleophile, THF, 110°C, 5 min.

Table 1. Synthesis of [^{11}C]**4b** and [^{11}C]**3b** labelled in the carbonyl position via Pd(0)-mediated aminocarbonylation

Entry	Product	Aryl halide (mM)	$Pd(PPh_3)_4$ (mM)	Diethyl-amine (mM)	*T*(°C)	[^{11}C]O-conv. (%)[a,b]	RCP (%)[b,c]
		4a					
1	[^{11}C]**4b**	14	12	100	25 to 140	91	3
2	[^{11}C]**4b**	14	5.0	100	130	87	9
3	[^{11}C]**4b**	9.0	9.0	1000	120	93	34
4	[^{11}C]**4b**	9.0	9.0	500	120	93	34
5	[^{11}C]**4b**	6.9	9.0	500	110	89	16
6	[^{11}C]**4b**	9.0	9.0	500	110	92 ± 1 (2)	44 ± 1 (2)
7	[^{11}C]**4b**	9.0	9.0	500	100	93 ± 1 (2)	38 ± 1 (2)
8	[^{11}C]**4b**	9.0	9.0	500	90	95	44
9	[^{11}C]**4b**	9.0	9.0	500	80	91	35
10	[^{11}C]**4b**	18	9.0	43	70	93	10
		3a					
11	[^{11}C]**3b**	18	9.0	43	90	89	6
12	[^{11}C]**3b**	9.0	9.0	500	110	91	10
13	[^{11}C]**3b**	4.5	9.0	500	110	78	2

Standard conditions were helium-carrier-added [^{11}C]carbon monoxide (10–100 nmol) in a 200-µl autoclave pressurized to 35 MPa with a solution of aryl halide **4a** or **3a**, $Pd(PPh_3)_4$, diethylamine in THF for 5 min at a specified temperature.
[a]Decay-corrected conversion yield of [^{11}C]carbon monoxide to non-volatile products remaining in the reaction mixture after evaporation of solvent by purging with nitrogen.
[b]The numbers in brackets indicate the number of experiments.
[c]Radiochemical purity of the crude product as the product of the decay-corrected product peak fraction from the analytical radio chromatogram and the [^{11}C]O-conversion.

Table 2. Synthesis of three analogues [^{11}C-*carbonyl*]-AT_2-R ligands via Pd-mediated aminocarbonylation

Product	Amine (mM)	cLogP[a]	$CLogD_{7.4}$[a]	K_i (nM)[b]		[^{11}C]O-conv. (%)[c,d]	RCP (%)[d,e]	Isolated RCY (%)[f,g]
				AT_2	AT_1			
[^{11}C]**4b**	Diethylamine	5.94	5.00	3.0 ± 0.3	> 10 000	92 ± 1 (2)	44 ± 1 (2)	16
[^{11}C]**4c**	Isopropylamine	5.77	4.84	2.6 ± 0.2	> 10 000	82	49	29
[^{11}C]**4d**	Benzylamine	6.73	5.79	1.0 ± 0.08	> 10 000	76	58	36

Standard conditions were helium-carrier-added [^{11}C]carbon monoxide (10–100 nmol) in a 200-µl autoclave pressurized with a solution of aryl-iodide **4a**, $Pd(PPh_3)_4$, amine in THF to 35 MPa for 5 min at 110°C.
[a]Calculated using Marwin Online software.
[b]Data from reference.[11] Binding assay relying on displacement of [^{125}I] Ang II from pig uterus myometrium (AT_2) and rat liver membranes (AT_1).
[c]Decay-corrected conversion yield of [^{11}C]carbon monoxide to non-volatile products remaining in the reaction mixture after evaporation of solvent by purging with nitrogen.
[d]The numbers in brackets indicate the number of experiments.
[e]Radiochemical purity of the crude product as the product of the decay-corrected product peak fraction from the analytical radio chromatogram and the [^{11}C]O-conversion.
[f]Decay-corrected radiochemical yield based on the initial amount of radioactivity at the start of the synthesis and the radioactivity of the isolated product. When the reaction mixture was transferred from the micro-autoclave to an evacuated vial, the radioactivity in the vial was measured. The radioactive residues left in the micro-autoclave were estimated to be less than 1%. Hence, the amount of initial radioactivity of [^{11}C]carbon monoxide could be determined.
[g]Radiochemical purity was > 97% in all experiments.

inhibitor.[29] The optimized reaction conditions for [^{11}C]**4b** (Table 1, Entry 6) were applied using other amine nucleophiles to synthesize the analogues [^{11}C]**4c** and [^{11}C]**4d** (Table 2). By this three-component combinatorial approach, large numbers of potential PET tracers may be synthesized for initial autoradiography screening without the need for tedious optimization procedures for each compound. This work flow has the potential to speed-up the development and SAR of new PET tracers. Although the three compounds [^{11}C]**4b–d** have comparable affinity and selectivity profiles, compound [^{11}C]**4c** was subjected to further biological investigation due to its favourable lower lipophilicity.

Specific activity

Specific activity of a PET tracer is important to avoid pharmacological effects on low abundance receptors and in order not to perturb the system under observation in

accordance with the tracer concept. As carbon monoxide is much less abundant in the atmosphere than carbon dioxide, isotopic dilution from stable carbon isotopes can be kept at a minimum by the use of online-produced [^{11}C]carbon monoxide. A 5 μAh bombardment resulted in 10.0 GBq [^{11}C]carbon monoxide at 7 min from end of bombardment (EOB) which was reacted with **4a**, Pd(0), and benzylamine. After 42 min from EOB, 1.10 GBq of the product [^{11}C]**4d** was isolated in 36% decay-corrected radiochemical yield (Table 2). The volume and concentration was measured to determine the amount of product (10 nmol). This corresponds to a specific activity of 110 Bq · μmol^{-1}. Following a 12-μAh bombardment, the specific activity of compound [^{11}C]**4c** was determined to 180 GBq · μmol at 47 min from EOB. Compound [^{11}C]**4c** formulated as a 1.2-μM solution in phosphate buffer pH 7.2 and appeared to be stable up to 3 h from EOB as determined by using analytical UV-radio-HPLC.

In vitro autoradiography

Using *in vitro* autoradiography on a number of frozen organ sections from rat and pig, specific [^{11}C]**4c** binding (affected by excess of unlabelled **4c**) was observed in the pancreas and kidney cortex and, also to some degree in the pig adrenals and rat brain (Figure 2). In rat heart and liver, we could not observe any specific binding (Figure 2), and in pig uterus and rat lung, we could not detect any binding at all (data not shown).

Organ distribution in rat

The distribution of [^{11}C]**4c** in dissected male and female rat organs is shown as standardized uptake values (SUVs) in Figure 3. No significant difference in the distribution was found between female and male rats. Very high radioactivity was found in the liver. Substantial radioactivity was also detected in the kidney, in the walls of the intestines, and in the adrenal glands. Very low radioactivity was noticed in most other organs, including the sexual organs of both genders.

Animal PET study

In the animal study, PET revealed excellent whole body image quality, resulting in high quality time–activity curves (TACs) (Figures 4 and 5). High accumulation was seen in the liver and in the urinary bladder, and a reversible uptake was also observed in the kidneys, which peaked already after 1 min after injection. The series of dynamic whole body scans revealed a reversible uptake of [^{11}C]**4c** in the kidney that peaked already at 1 min after injection and high accumulation and retention in the liver and urinary bladder.

Discussion of the biological evaluation

Three different methods have been used to evaluate the binding and distribution of [^{11}C]**4c** in animals, one *in vitro* and two *in vivo*. The *in vitro* autoradiographic analysis revealed that the ligand could bind specific sites in some organs, and especially in the pancreas and cortex of the kidney. The two *in vivo* methods, organ distribution and animal PET studies, both showed an intense accumulation in the liver, a region where there was virtually no specific binding *in vitro*. The rapid and high accumulation of radioactivity in the urinary bladder in the PET study also indicates rapid metabolism. Thus, it can be suggested that the accumulation of benzamide compound [^{11}C]**4c** (and ^{11}C-containing **4c**-metabolites) in the liver and in the urine *in vivo* is mainly due to fast metabolism. High uptake of [^{11}C]**4c** in liver excluded the possibility to measure tracer kinetics in the pancreas in the animal PET study. The accumulation of [^{11}C]**4c** according to the rat organ distribution (Figure 3) is generally in accordance with previous reports on the distribution of AT_2 receptors,[30] although the observed activity in metabolizing organs are very high. The organ distribution study did not show any marked uptake of [^{11}C]**4c** in the pancreas, a

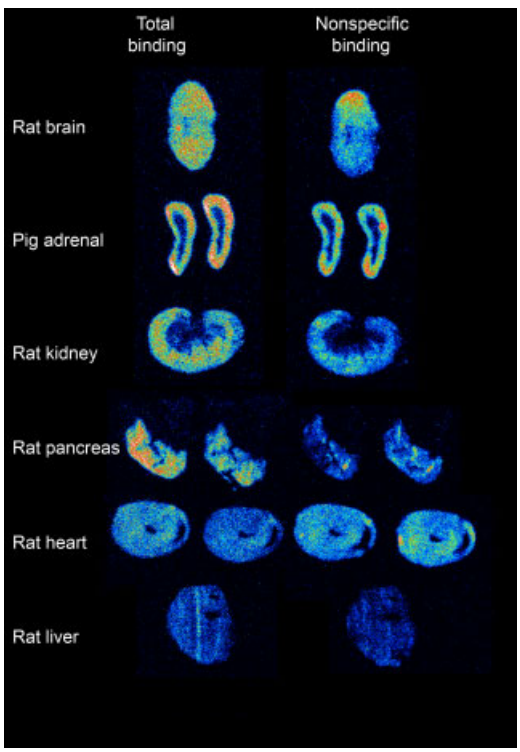


Figure 2. The sections (25 μm) were incubated with [^{11}C]**4c** (0.3 nM; 0.06 MBq ml^{-1}) in Tris–HCl buffer with salts for 40 min at room temperature. The nonspecific binding was defined as binding in the presence of 3.5 μM unlabelled **4c**. The slides were washed, dried, and exposed to phosphor imaging plates over night.

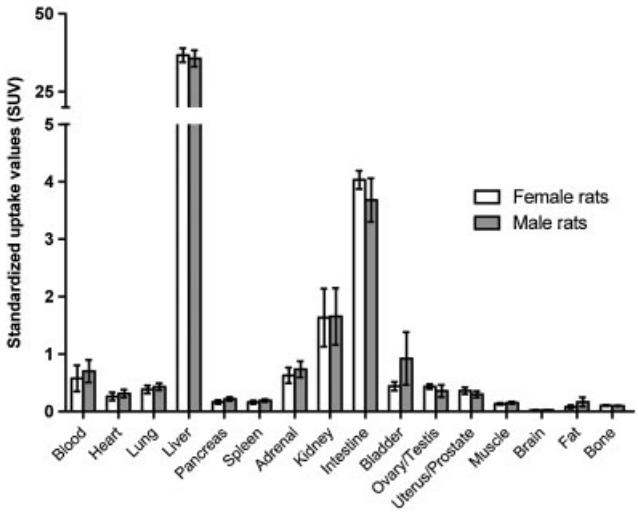


Figure 3. Three male and three female rats were injected intravenously with 17–18 MBq per animal of [^{11}C]**4c** (for details, see Table 3). The animals were killed after 40 min, and the selected organs were dissected out, weighed, and the radioactivity was measured in an automated gamma counter. The SUVs are calculated as described in the Experimental Section.

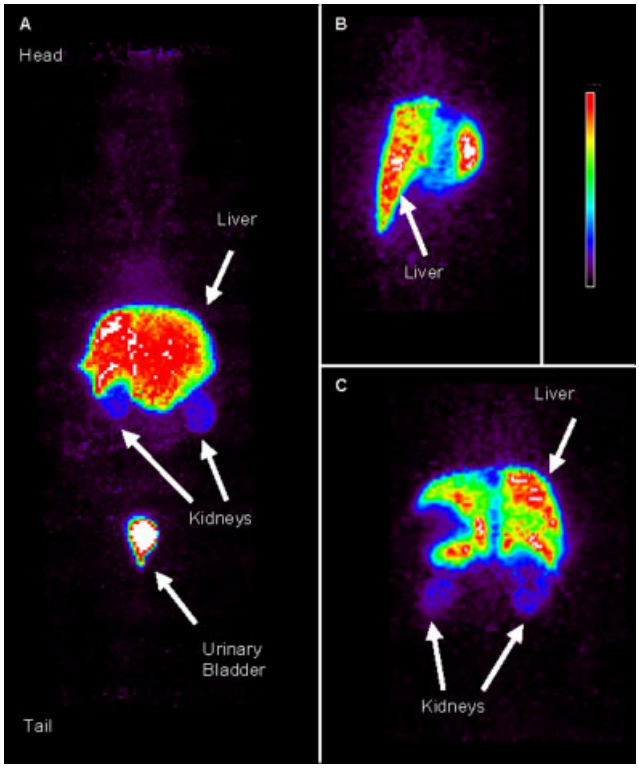


Figure 4. Small animal PET scan of a female rat injected with 12.2 MBq of [^{11}C]**4c**. For methodological details, see Experimental Section. (a) Whole body view showing liver, urinary bladder and kidneys. (b) Enlargement of liver (sagittal view). (c) Enlargement of liver and kidneys (coronary view).

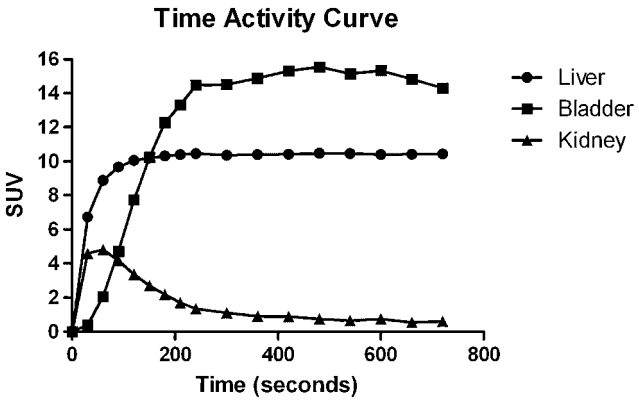


Figure 5. TACs of radioactivity in the liver, bladder and kidney in female rat after intravenous injection of 12.2 MBq [^{11}C]**4c** The calculations are described in the Experimental Section.

region that showed marked specific binding *in vitro*. Although AT_2 receptors have been shown be located in the sexual organs,[1] no clear accumulation of radioactivity was observed in any of the animal studies. Similarly, no uptake in the brain was revealed, which might be explained by poor blood-brain barrier penetration, or by the action of active transporters (e.g. P-glycoproteins).[31]

Experimental section

General

^{11}C was prepared by the ^{14}N(p,α)^{11}C nuclear reaction using 17 MeV proton beam produced by a Scanditronix MC-17 Cyclotron at Uppsala Imanet, GE Healthcare and obtained as [^{11}C]carbon dioxide. The target gas used was nitrogen (AGA Nitrogen 6.0) containing 0.05% oxygen (AGA Oxygen 4.8). The radioactivity was concentrated on a silica column at 196°C, transferred online via a helium flow to the hotcell and once again concentrated on a silica column at 196°C. The helium-carrier-added [^{11}C]carbon dioxide was reduced to [^{11}C]carbon monoxide over zinc at 400°C, concentrated on silica at 196°C, and transferred to a stainless steel micro-autoclave (200 µl) according to a previously described technique.[32,33]

The identities of the ^{11}C-labelled compounds were determined by using analytical HPLC using authentic samples as references. Analytical HPLC was performed on a Beckman system, equipped with a Beckman 126 pump, a Beckman 166 UV detector in series with a Bioscan β^+-flow count detector, and a Beckman Ultrasphere ODS dp 5µ column (250 × 4.6 mm). A Gilson 231 XL was used as auto injector. Flow: 1 ml min^{-1}. Mobile phase: (A) 50 mM Ammonium formate pH 3.5, (B) Acetonitrile, (C) Methanol. When the product was eluted, the column was washed with 95% organic phase. Further characterization of the purified labelled products was made using a Waters Quattro Premier triple quadrupole mass spectrometer with electrospray ionization operated in positive mode coupled to a Waters analytical HPLC system. Purification with semipreparative HPLC was performed on a similar Beckman system equipped with a Genesis C18 120 4µ (250 × 10 mm). Flow: 5 ml min^{-1}. Mobile phase: (A) 50 mM Ammonium formate pH 3.5, (B) Acetonitrile, (C) Methanol.

^{1}H and ^{13}C NMR spectra were recorded on a Varian 400 MHz spectrometer at 25°C and chemicals shifts are given in ppm (δ) using $CHCl_3$ (7.26 ppm for ^{1}H and 77.16 for ^{13}C) or acetone-d_6 (2.09 ppm for ^{1}H and 30.92 for ^{13}C) as internal standard. RP-LC-MS was carried out on a Gilson HPLC instrument equipped with an analytical column Zorbax SB C10 (4.6 × 50 mm, 5 µm; Agilent Technologies) or a preparative column Zorbax SB-C8 (21.2 × 150 mm, 5 µm; Agilent Technologies) and a Finnigan quadropole mass spectrometer, eluting with H_2O/MeCN/0.05% formic acid (4 ml min^{-1}). Column chromatography was carried out using silica gel (0.040–0.063 mm; Merck) and thin-layer chromatography was performed on precoated aluminium plates (silica, F_{254}) and visualized with ultraviolet light at 254 nm. Elementary analyses were carried out by Analytische Laboratorien (Lindlar, Germany). Dry dichloromethane was distilled over calcium hydride and dry tetrahydrofuran over sodium and benzophenone. Synthesis of intermediates and references **2c**, **2d**, **3c**, **3d**, **4c,** and **4d** are previously described.[11] All other chemicals were purchased from commercial suppliers and used without further purification.

Synthesis of precursors and reference compounds

2-(N-tert-Butylsulphamoyl)-4-isobutylcyclopenta-1,4-dienylboronic acid (1)[24]

To a solution of *N*-(*tert*-butyl)-5-isobutylthiophene-2-sulphonamide (948 mg, 3.44 mmol) in dry THF was added dropwise *n*-BuLi (5.4 ml, 8.6 mmol) as 1.6 M in hexanes under nitrogen atmosphere at 78°C and stirred for 1 h whereupon the temperature was raised to −30°C and maintained for 3 h. The temperature was decreased to 40°C and triisopropylborate (1.3 ml, 5.4 mmol) was added dropwise over 10 min and the reaction mixture was allowed to

warm to room temperature and stirred overnight. The reaction mixture was cooled to 0°C and acidified to pH 2 with 2 M HCl, prior to extraction with EtOAc and washed with brine and water. The organic layer was dried ($MgSO_4$), filtered and evaporation of the solvent yielded the crude product **2** as a brown oil with an estimated purity of >95% as confirmed by ^{1}H-NMR, which was used in the next step without further purification. LC-MS (ESI+) *m/z*: 320.1 ($M+H^+$).

*General procedure of the Suzuki-coupling exemplified by N-(tert-butyl)-3-(4-iodophenyl)-5-isobutylthiophene-2-sulphonamide (**2a**)*

A Smith Process Vial™ (2–5 ml) was charged with crude boronic acid **1** (152 mg, 0.46 mmol), 1,4-diiodobenzene (188 mg, 0.57 mmol), 2.0 ml DME, 0.5 ml ethanol, 0.5 ml water and 0.5 ml Na_2CO_3 (2 M, 1 mmol) and $Pd(PPh_3)_4$ (21.6 mg, 0.020 mmol) and microwave irradiated for 10 min at 110°C. The reaction mixture was extracted with CH_2Cl_2 and washed with brine and water, prior to drying ($MgSO_4$) and evaporation of solvent. The crude product was purified by column chromatography (first pure isohexane then isohexane:EtOAc 9:1) as white crystals in quantitative yield (221 mg, 0.46 mmol) based on boronic acid **1**.^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.76 (2 H, AA′XX′, Ar-*H*), 7.35 (2 H, AA′XX′, Ar-*H*), 6.72 (1 H, s, Ar-*H*), 4.15 (1 H, br s, N*H*), 2.67 (2 H, dd, *J* = 7.1, 0.8 Hz, CH_2), 1.96–1.86 (1 H, *m*, *J* = 6.7 Hz, C*H*), 1.03 (9 H, s, 3 × CH_3), 0.97 (6 H, d, *J* = 6.7 Hz, 2 × CH_3) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ: 149.1, 142.5, 137.9, 137.1, 134.8, 131.2, 129.1, 94.8, 55.1, 39.6, 30.9, 29.9, 22.5 ppm. Found: C, 45.42; H, 5.17; N, 2.87. Calc. for $C_{18}H_{24}INO_2S_2$: C, 45.28; H, 5.17; N, 2.87%.

*4-(2-(N-tert-Butylsulphamoyl)-5-isobutylthiophen-3-yl)-N,N-diethylbenzamide (**2b**). Same procedure as for **2a***

Column chromatography (first isohexane, then 4:1 isohexane: EtOAc, then 2:1 isohexane:EtOAc) yield 66% as off-white crystals. ^{1}H NMR (400 MHz, acetone-d_6) δ: 7.75 (2 H, AA′XX′, Ar-*H*), 7.47 (2 H, AA′XX′, Ar-*H*), 6.99 (1 H, *m*, *J* = 0.9 Hz, Ar-*H*), 5.97 (1 H, br s, N*H*), 3.66–3.22 (4 H, *m*, 2 × CH_2), 2.80 (2 H, dd, *J* = 7.2 Hz, 0.9 Hz, CH_2), 2.04–1.97 (1 H, *m*, C*H*), 1.27 (6 H, *m*, N(CH_2CH_3)$_2$), 1.08 (9 H, s, 3 × CH_3), 1.03 (6 H, d, *J* = 6.6 Hz, 2 × CH_3) ppm. ^{13}C NMR (100 MHz, acetone-d_6) δ: 170.1, 149.0, 142.3, 137.6, 137.1, 136.0, 129.4, 129.3, 126.9, 54.9, 43.5, 39.7, 39.5, 30.8, 29.8, 22.5, 14.5, 13.2 ppm.

*General procedure of the deprotection exemplified by 3-(4-iodophenyl)-5-isobutylthiophene-2-sulphonamide (**3a**)*

To a cooled (0°C) solution of **2a** (65.2 mg, 0.14 mmol) in dry CH_2Cl_2 (5 ml) under a nitrogen atmosphere BCl_3 was added (1 ml, 0.55 mmol) over 10 min. After stirring for 1 h at room temperature, the reaction mixture was co-evaporated four times with $CHCl_3$. The crude product was purified by column chromatography (first pure isohexane, then 3:1 isohexane: EtOAc) and isolated as white crystals in 63% yield (33 mg, 0.08 mmol). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.79 (2 H, AA′XX′, Ar-*H*), 7.35 (2 H, AA′XX′, Ar-*H*), 6.77 (1 H, s, Ar-*H*), 4.74 (2 H, br s, NH_2), 2.70 (2 H, dd, *J* = 7.1, 0.8 Hz, CH_2), 1.94 (1 H, m, *J* = 6.7 Hz, C*H*), 1.01 (6 H, d, *J* = 6.7 Hz, 2 × CH_3) ppm.

*N,N-Diethyl-4-(5-isobutyl-2-sulphamoylthiophen-3-yl)benzamide (**3b**). Same procedure as for **3a***

The crude product was used in the next step without further purification. ^{1}H NMR (400 MHz, acetone-d_6) δ: 7.73 (2 H, AA′XX′, Ar-*H*), 7.44 (2 H, AA′XX′, Ar-*H*), 6.99 (1 H, *m*, Ar-*H*), 6.71 (2 H, br s, NH_2), 3.53–3.40 (4 H, *m*, N(CH_2CH_3)$_2$), 2.78 (2 H, dd, *J* = 7.1, 0.8 Hz, CH_2), 1.99 (1 H, *m*, *J* = 6.6 Hz, C*H*), 1.21 (6 H, *m*, N(CH_2CH_3)$_2$), 1.03 (6 H, d, *J* = 6.6 Hz, 2 × CH_3) ppm. ^{13}C NMR (100 MHz, acetone-d_6) δ: 171.8, 148.7, 143.8, 139.2, 138.9, 137.2, 131.4, 130.7, 127.9, 40.1, 32.0, 23.2, 15.2, 13.9 ppm.

*General procedure for deprotection and acylation exemplified with butyl 3-(4-iodophenyl)-5-isobutylthiophen-2-ylsulphonylcarbamate (**4a**)*

Compound 2a (76 mg, 0.16 mmol) was dissolved in dry CH_2Cl_2 (5 ml) and cooled to 0°C under nitrogen. To the solution was added BCl_3 (1 ml) as 1.0 M in hexane. After 1 h, the reaction mixture was co-evaporated four times with $CHCl_3$. The residue was dissolved in CH_2Cl_2 (5 ml), thereafter triethylamine (88 µl, 0.64 mmol) and *n*-butyl chloroformate (24.5 µl, 0.19 mmol) was added and the reaction mixture was stirred for 2 h at room temperature. The reaction mixture was diluted with CH_2Cl_2 and washed with water and brine. The organic layer was dried ($MgSO_4$) and evaporated prior to purification by column chromatography (first pure isohexane, then 3:1 isohexane: EtOAc), to yield **4a** as white crystals (38 mg, 0.07 mmol, 44%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.75 (2 H, AA′XX′, Ar-*H*), 7.34 (1 H, br s, N*H*), 7.21 (2 H, AA′XX′, Ar-*H*), 6.74 (1 H, *m*, Ar-*H*), 4.05 (2 H, *t*, *J* = 6.5 Hz, OCH_2), 2.71 (2 H, d, *J* = 7.1, 0.7 Hz, Ar-CH_2), 1.94 (1 H, *m*, *J* = 6.6 Hz, C*H*), 1.52 (2 H, *m*, CH_2), 1.27 (2 H, *m*, *J* = 7.3 Hz, CH_2), 0.99 (6 H, d, *J* = 6.6 Hz, 2 × CH_3), 0.89 (3 H, *t*, *J* = 7.3 Hz, CH_3) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ: 152.2, 150.3, 145.6, 137.7, 133.7, 130.9, 129.5, 129.4, 95.2, 67.3, 39.5, 30.8, 30.6, 22.5, 19.0, 13.9 ppm. Found: C, 42.37; H, 4.57; N, 2.50. Calc. for $C_{19}H_{24}INO_4S_2 \cdot H_2O$: C, 42.30; H, 4.86; N, 2.60%.

*N-Butoxycarbonyl-3-[4-(N,N-diethylcarbamoyl)phenyl]-5-isobutylthiophene-2-sulphonamide (**4b**)*

Purification by using column chromatography (first pure isohexane, then 3:1 isohexane: EtOAc) and then RP-LC-MS yield 18% as white crystals. ^{1}H and ^{13}C NMR analyses were in agreement with earlier published data.

Labelling chemistry

*N-Butoxycarbonyl-3-[4-(N,N-diethyl[^{11}C]carbamoyl)phenyl]-5-isobutylthiophene-2-sulphonamide ([^{11}C]**4b**)*

To a 800-µl vial was added aryl halide **4a** (1.8 mg, 3.45 µmol) and *tetrakis*(triphenylphosphine)palladium(0) (4.0 mg, 3.45 µmol). The vial was capped and purged with argon before addition of 380 µl of THF. The mixture was heated at 70°C for 1 min and then kept at room temperature for approximately 10 min before the mixture was added to a purged vial containing diethylamine (20 µl, 14.35 mg, 200 µmol). The mixture was injected onto a loop (200 µl) and transferred to a 200-µl autoclave containing [^{11}C]carbon monoxide (10–100 nmol). The preheated autoclave was pressurized by THF using an HPLC to 35 MPa and kept at 110°C for 5 min. The reaction mixture was ejected into a capped and evacuated 5-ml vial, and the reactor was pressurized once more with THF and ejected, and the radioactivity was measured. The solvent was removed by purging with a flow of nitrogen at 70°C for approx 1 min, and the radioactivity was measured again. The residue was dissolved again by the addition of 1 ml acetonitrile and an aliquot was withdrawn and analysed using analytical UV-radio-HPLC. R_t = 8.2 min, isocratic 40%A, 60%B. The

crude product was purified using semipreparative HPLC. $R_t = 9.5$ min, isocratic 30% A, 70% B.

*N-Butoxycarbonyl-3-[4-(N-isopropyl[^{11}C]carbamoyl)phenyl]-5-isobutylthiophene-2-sulphonamide ([^{11}C]**4c**)*

Similar procedure as for [^{11}C]**4b**. Aryl halide **4a** (1.8 mg, 3.45 μmol) and *tetrakis*(triphenylphosphine)palladium(0) (4.0 mg, 3.45 μmol), isopropylamine (17 μl, 11.8 mg, 200 μmol). Analytical HPLC, $R_t = 8.9$ min, isocratic 30%A, 70%C. Semipreparative HPLC, $R_t = 7.7$ min, isocratic 30%A, 70%C. LC-MS-MS (ESI+) *m/z*: 503 ($M+Na^+$), 481 ($M+H^+$). For biological deliveries, the purified fraction was first concentrated using a stream of nitrogen and then diluted with phosphate buffer.

*N-Butoxycarbonyl-3-[4-(N-benzyl[^{11}C]carbamoyl)phenyl]-5-isobutylthiophene-2-sulphonamide ([^{11}C]**4d**)*

Similar procedure as for [^{11}C]**4b**. Aryl halide **4a** (1.8 mg, 3.45 μmol) and *tetrakis*(triphenylphosphine)palladium(0) (4.0 mg, 3.45 μmol), benzyl amine (24 μl, 21.4 mg, 200 μmol). Analytical HPLC, $R_t = 6.4$ min, isocratic 25%A, 75%C. Semipreparative HPLC, $R_t = 9.7$ min, isocratic 20%A, 80%C. LC-MS-MS (ESI+) *m/z*: 551 ($M+Na^+$), 528 ($M+H^+$).

Specific activity

Specific activity of [^{11}C]**4d** was determined following a 5-μAh bombardment. The volume of the purified product fraction was measured with a syringe, and the radioactivity was measured. An aliquot was withdrawn and the concentration was determined using analytical UV-radio-HPLC and a three point UV-calibration curve, inj. vol 50 μl. In the same manner, [^{11}C]**4c** was analysed following a 12-μAh bombardment.

In vitro autoradiography

Frozen sections (25 μm) were prepared in a cryomicrotome and placed on SuperFrost glass slides (SuperFrost® Plus; MenzelGläser, Germany). The slides were kept in a freezer (20°C) before use. At the start of the experiment, the slides were pre-incubated for 10 min in Tris–HCl buffer, pH 7.4, including salts (Tris 50 mM, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$). The slides were then transferred to containers containing [^{11}C]**4c** (0.03–0.3 nM; 0.006–0.06 MBq/ml) in buffer. In a duplicate set of containers, 3.5 μM unlabelled **4c** was added to block specific binding. After incubation for 40 min at room temperature, the slides were washed three times for 3 min each in buffer. The slides were dried in a heated (37°C) oven and exposed to phosphor imaging plates (Molecular Dynamics, USA) overnight. The incubated (20 μl) plates was pipetted onto a filter paper (standard activity area), which was exposed in parallel to the experimental sections. The plates were then scanned in a Phosphor Imager Model 400S using 100 μm pixel width (Molecular Dynamics, USA).

Animal ethics

The animals were handled according to the guidelines by the Swedish Animal Welfare Agency, and the experiments were approved by the local Ethics Committee for Animal Research, permit no: C234/5.

Organ distribution

To evaluate the uptake of [^{11}C]**4c** in normal tissues, the uptake of radioactivity in a number of rat organs was investigated. Male and female rats were injected intravenously with 17–18 MBq per animal (for details, see Table 3). The animals were killed after 40 min, and selected organs were dissected out. Blood, heart, lung, liver, pancreas, spleen, adrenal, kidney, intestine (without contents), urinary bladder, ovary or testis, muscle, brain, fat, bone (femur), and uterus or prostate were collected, weighed and the radioactivity was determined and corrected for radioactive decay in an automated gamma counter. Organ values were calculated as SUV, calculated as follows:

$$\mathrm{SUV} = \frac{\mathrm{ACT(Bq/g)}}{\mathrm{DOSE(Bq)/BW(g)}}$$

where ACT is the measured concentration of radioactivity corrected for physical decay, DOSE is the administered amount of radioactivity (Bq) and BW is the body weight of the animal (g).

PET scanning and image processing

Imaging was performed using the small animal PET system (eXplore Vista; GE Healthcare). In brief, the system has a transaxial field of view (FOV) of 6.7 cm and an axial FOV of 4.8 cm, achieving an in-plane spatial and volume resolution of approximately 1.4 mm and 2.9 mm^3, respectively.

Immediately preceding the administration of the tracer, the animal was placed in a plastic container and anaesthetized with 3.5–4.0% isoflurane. This concentration was kept during administration, and lowered to 2.0–3.0% isoflurane via a mask during the PET experiment. The animal was placed in the prone position in the scanner gantry, and radiotracer (12.2 MBq) was injected via the tail vein. Image acquisition and radiotracer injection started simultaneously, and a dynamic sequence (16 frames: 8×30 s and 8×60 s) was acquired over 60 min.

Image reconstruction and TACs

Transaxial images were reconstructed by a use of Fourier rebinning with two-dimensional ordered-subset expectation maximization 3D FORE/2D OSEM (2 iteration and 16 subsets). Image data were corrected for attenuation and scatter. For an organ-based analysis, the liver, bladder, and left kidney were identified on PET images, and regions of interest (ROIs) associated with these organs were drawn. ROIs initially indicate values in counts per cubic centimetre. The counts were converted to a radioactivity concentration using a cross-calibration factor (CCF):

$$\text{Tissue radioactivity concentraion} = \frac{\text{Counts}}{\text{cm}^3} \times \text{CCF (Bq/Count)}$$

SUV was calculated according to the formula in previous section. Finally, a TAC was generated.

Table 3. Details on the animals (mean ± SD, $n = 3$ per gender)

	Weight (g)	Given dose (MBq)	Given dose/weight ($kBq \cdot g^{-1}$)	Given dose/weight ($pmol \cdot g^{-1}$)
Females	298 ± 8	17.1 ± 1.3	57.3 ± 4.6	0.88 ± 0.087
Males	447 ± 15	18.0 ± 2.1	40.3 ± 5.3	0.81 ± 0.16

Conclusions

As a part of our ongoing investigation of the angiotensin II receptors using PET, we have ^{11}C-labelled three analogues of selective and high-affinity nonpeptide ligands of the AT_2 receptor using [^{11}C]carbon monoxide and an aminocarbonylation protocol. By screening different reaction conditions, the highest radiochemical purities of the crude products were obtained using a high concentration of amine and a 1:1 ratio of aryl halide:palladium complex at 90–110°C. After purification, isolated AT_2 tracers were obtained with high specific activity and in sufficient radiochemical yields for biological testing. The biological evaluation of [^{11}C]**4c** in the rat indicate rapid distribution and elimination but also weak binding to the heart, lungs, adrenal glands, ovaries and uterus, partly supporting previous studies using AngII, immunohistochemistry, AT_2 receptor mRNA distribution etc. It should be emphasized that further development of more metabolically stable PET tracers, and the start up of additional reference studies with [^{125}I]AngII and selective AT_1 and AT_2 blockers is necessary to improve and validate the PET methodology as a tool for AT_2 receptor studies. Owing to different elimination kinetics in different species, further *in vivo* studies in other species are needed. Finally, we look forward to continue our search for a more suitable PET-tracer to study the AT_2 expression in myocardial infarction models and foetal tissues.

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