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Synthesis and evaluation of (S)-2-(2-[^{18}F]fluoroethoxy)-4-([3-methyl-1-(2-piperidin-1-yl-phenyl)-butyl-carbamoyl]-methyl)-benzoic acid ([^{18}F]repaglinide): a promising radioligand for quantification of pancreatic β-cell mass with positron emission tomography (PET)

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Abstract

^{18}F-labeled non-sulfonylurea hypoglycemic agent (S)-2-(2-[^{18}F]fluoroethoxy)-4-((3-methyl-1-(2-piperidin-1-yl-phenyl)-butylcarbamoyl)-methyl)-benzoic acid ([^{18}F]repaglinide), a derivative of the sulfonylurea-receptor (SUR) ligand repaglinide, was synthesized as a potential tracer for the non-invasive investigation of the sulfonylurea 1 receptor status of pancreatic beta-cells by positron emission tomography (PET) in the context of type 1 and type 2 diabetes. [^{18}F]Repaglinide could be obtained in an overall radiochemical yield (RCY) of 20% after 135 min with a radiochemical purity higher than 98% applying the secondary labeling precursor 2-[^{18}F]fluoroethyltosylate. Specific activity was in the range of 50–60 GBq/μmol. Labeling was conducted by exchanging the ethoxy-moiety into a 2-[^{18}F]fluoroethoxy group. To characterize the properties of fluorinated repaglinide, the affinity of the analogous non-radioactive ^{19}F-compound for binding to the human SUR1 isoform was assessed. [^{19}F]Repaglinide induced a complete monophasic inhibition curve with a Hill coefficient close to 1 (1.03) yielding a dissociation constant (K_D) of 134 nM. Biological activity was proven via insulin secretion experiments on isolated rat islets and was comparable to that of repaglinide. Finally, biodistribution of [^{18}F]repaglinide was investigated in rats by measuring the concentration of the compound in different organs after i.v. injection. Pancreatic tissue displayed a stable accumulation of ≈0.12% of the injected dose from 10 min to 30 min p.i.. 50% of the radioactive tracer could be displaced by additional injection of unlabeled repaglinide, indicating that [^{18}F]repaglinide might be suitable for in vivo investigation with PET.

Keywords: [^{18}F]Repaglinide; Sulfonylurea receptors; β-Cell imaging agents; Diabetes; PET

1. Introduction

Diabetes mellitus comprises a heterogeneous group of disorders characterized by high blood glucose levels. Two major types of diabetes mellitus have been defined: type 1 and type 2 diabetes. Although hyperglycemia is the common denominator of both type 1 and type 2 diabetes, the etiology and pathophysiology of these syndromes are distinct. Type 1 diabetes is a chronic autoimmune disease characterized by the selective destruction of insulin-producing β-cells of the islets of Langerhans. When autoimmune destruction affects more than 90% of the β-cell mass, the resulting insulin deficiency culminates into the development of overt hyperglycemia. In type 2 diabetes, on the other hand, the pancreatic β-cells are initially intact, and the disease is associated with insulin resistance and loss of β-cell function, and eventual insulin-dependency [1,2].

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Insulin secretion is regulated by the membrane potential of the β-cell, which depends on the activity of ATP-sensitive K^+ channels (K_{ATP} channels) in the plasma membrane [3]. Closure of K_{ATP} channels due to a rise of the cytoplasmic ATP/ADP ratio results in a depolarization of the membrane and in opening of voltage-sensitive Ca^{2+} channels. The increase in cytoplasmic Ca^{2+} stimulates the exocytosis of insulin.

K_{ATP} channels are composed of a small inwardly rectifying K^+ channel subunit (Kir6.1 or Kir6.2) plus a sulfonylurea receptor (SUR1, SUR2A, or SUR2B) belonging to the ATP-binding cassette superfamily [4]. SURs represent the target for hypoglycemic sulfonylureas, a group of well known antidiabetic agents which have been in clinical use for years, as well as for repaglinide, a novel fast acting prandial glucose regulator with a short plasma half-life (<1 h) [5–7]. Repaglinide is the first member of the carbamoylmethylbenzoic acid chemical family to be used in a clinical setting, being a new chemical class of insulin secretagogues with an insulin release profile which is very different to sulfonylureas like glibenclamide [8]. The development of radioligands which bind avidly to subtypes of K channels is an important task for K channel research. For example radioiodinated charybdotoxin labels high-conductance calcium-activated K channels [9] and [^{3}H]glibenclamide [10] and ^{125}I-labeled glibenclamide [11] label ATP-inhibited K channels. Recently, enantiomerically pure [^{14}C]repaglinide was used to obtain in vivo data about its metabolism and way of excretion [12]. A major drawback of this study, apart from low specific activity (2–50 Ci/mmol) is the use of carbon-14 whose radiation characteristics (short range β^- particle (200 keV energy), half-life $t_{1/2}$=5730 a) preclude a non-invasive investigation. Radiolabeled sufonylureas such as ^{18}F-labeled glibenclamide derivatives have been described in literature [13] and have been proven to be unsuitable for β-cell quantification, most recently by Shiue and co-workers [14]. Positron emission tomography (PET) is a novel imaging technique to quantitatively assess biodistribution of radiolabeled pharmaceuticals non-invasively in humans and animals in vivo. This method is predestined to quantify and visualize the receptor status of diverse receptor systems. The dopaminergic system of the brain, e.g., has been intensively investigated by means of radioactively labeled 2-[^{18}F]fluoro-DOPA and a variety of high-affinity D2-receptor ligands [15]. However, non-invasive investigations may become available by using a radioactive analogue with high specific activity and labeled with a positron emitting radionuclide, e.g. ^{11}C or ^{18}F. Labeling with [^{11}C]carbon would have the advantage of obtaining a structurally unchanged molecule [16]. For fluorine-18, the low energy of the positron results in the highest possible resolution for PET imaging and the nearly 2 h half-life allows for a more complex synthesis to be carried out within the decay time of the radioisotope and acquisition of in vivo data can become extended. Thus, [^{18}F]-fluorine is the most attractive radionuclide for PET imaging [17]. As the electronegative nature of fluorine can alter the electron distribution in a way that may alter the binding properties of the molecule, the biological activity of a fluorinated repaglinide derivative must be tested in vitro. If biological activity is retained, a ^{18}F-labeled repaglinide derivative with high specific activity might become a valuable tool for the visualization and quantification of human pancreatic β-cell mass in vivo.

2. Materials and methods

All reagents were purchased from Acros-Organics BVBA (Belgium), Merck KGaA (Germany), Sigma-Aldrich Chemie GmbH (Germany) or Lancaster Synthesis GmbH (Germany). Analytic TLC was performed using plates from Merck KGaA (Silica gel 60 F_{254}, thickness 0.25 mm). Column chromatography was performed with silica gel (Si-60, Merck KGaA). All solvents for column chromatography were p.a. grade. ^{1}H, ^{13}C, and ^{19}F nuclear magnetic resonance (NMR) spectra were recorded using a DRX 400 spectrometer (Bruker Analytik GmbH). Chemical shifts are quoted in δ (ppm) downfield from TMD as an internal standard. Mass spectrometry spectra were obtained on a MAT90 spectrometer (Finnigan). Elemental analyses were performed with an EL2 system (Elementar vario). High performance liquid chromatography (HPLC) was performed with a Sycam S1100 system, UV detection was obtained using an UV detector Sycam S3200. Detection of radioactivity was performed using an Instant Imager (Packard Canberra) for radio-TLC and detection of radio-HPLC was performed using a NaI-radiodetector (Packard Canberra). [^{18}F]Fluoride as produced via the ^{18}O(p,n)^{18}F reaction was purchased from different institutions. Purification of the labeling precursor 2-[^{18}F]fluoroethyl tosylate and [^{18}F]repaglinide was achieved using HPLC (column: LiChrospher 100 RP18- 5 EC, 250 mm×10 mm).

2.1. (S)-3-Methyl-1-(2-(1-piperidinyl)phenyl)-butylamine 7

(S)-3-Methyl-1-(2-(1-piperidinyl)phenyl)-butylamine was synthesized and characterized as previously described [18]. Enantiomeric separation was accomplished via fractionated crystallization of the salt of 3-methyl-1-(2-(1-piperidinyl)phenyl)-butylamine with L(-)-N-acetylglutamic acid and verified by chiral HPLC [18].

2.2. 2-Hydroxy-4-methyl-benzoic acid methyl ester 2

To a stirred solution of 2-hydroxy-4-methyl-benzoic acid **1** (52.6 g, 350 mmol) in methanol (500 mL) concentrated sulphuric acid was added (16 mL). After refluxing for 16 h the mixture was reduced in volume and extracted with diethylether (3×150 mL). The combined ether phases were evaporated in vacuo to yield **2** as white needles, yield 60%.

^{1}H NMR (DMSO-d_6) δ 10.5 (*s*, ^{1}H), 7.7 (*d*, ^{1}H, J=8.1),

6.7 (*s*, ^{1}H), 6.6 (*d*, ^{1}H, *J*=8.1 Hz), 2.2 (*s*, ^{3}H); ^{13}C NMR (DMSO-d_6) δ 169.6, 160.7, 147.1, 129.9, 120.8, 117.7, 110.2, 52.5, and 21.5; FD-MS: *m/z* (%)=166.2 (100%); Anal. $C_9H_{10}O_3$ (calculated): C 64.88%, H 6.12% (C 65.05%, H 6.07%).

2.3. 4-Bromomethyl-2-hydroxy-benzoic acid methyl ester 3

To a solution of **2** (10 g, 60 mmol) in tetrachlorocarbon (50 mL) NBS (9.8 g, 55 mmol) and α-α′ azobisisobutyronitrile (AIBN) (0.8 g, 5 mmol) were added and the mixture was refluxed overnight. The mixture was filtered and washed with tetrachlorocarbon (3×10 mL). The filtrate was evaporated in vacuo. The crude residue was dissolved in hot petrolether and crystallized as a yellow solid, yield 59%. ^{1}H NMR (DMSO-d_6) δ 10.5 (*s*, ^{1}H), 7.6 (*d*, ^{1}H, *J*=8.1 Hz), 7.0 (*d*, ^{1}H, *J*=1.6 Hz), 6.9 (*dd*, ^{1}H, *J*=1.6 Hz, *J*=8.1 Hz), 4.6 (*s*, ^{2}H), 3.8 (*s*, ^{3}H); ^{13}C NMR (DMSO-d_6) δ 168.9, 160.1, 145.8, 130.7, 120.5, 118.1, 113.1, 52.7, and 33.1; FD MS: *m/z* (%)=244.2 (44.6%), 246.2 (48.0%); Anal. $C_9H_9BrO_3$ (calculated): C 44.23%, H 3.61% (C 44.11%, H 3.70%).

2.4. 4-Cyanomethyl-2-hydroxy-benzoic acid methyl ester 4

To a solution of NaCN (2.95 g, 60 mmol) and N-benzyl-tri-*n*-butylammoniumchloride (0.72 g, 2.3 mmol) in water (60 mL) a solution of **3** (12.3 g, 50 mmol) in CH_2Cl_2 (50 mL) was added dropwise at 20°C. After the mixture was stirred for 48 h at 20°C, the organic phase was separated, washed with water and evaporated in vacuo. The residue was triturated with petrolether to give **4** as a yellow solid, yield 85%. ^{1}H NMR (DMSO-d_6) δ 10.5 (*s*, ^{1}H), 7.7 (*d*, ^{1}H, *J*=8.1 Hz), 6.9 (*d*, ^{1}H, *J*=1.6 Hz), 6.8 (*dd*, ^{1}H, *J*=1.6, *J*=8.1 Hz), 4.0 (*s*, ^{2}H), 3.8 (*s*, ^{3}H).

^{13}C NMR (DMSO-d_6) δ 168.9, 160.3, 139.3.130.9, 119.3, 118.7, 117.0, 112.7, 52.6, and 22.7; FD MS: *m/z* (%)=191.2 (100%); Anal. $C_9H_9NO_3$ (calculated): C 62.59%, H 4.62%, N 7.24% (C 62.82%, H 4.74%, N 7.33%).

2.5. 2-Hydroxy-4-methoxycarbonylmethyl-benzoic acid methyl ester 5

4 (9.6 g, 50 mmol) was treated continuously with gaseous HCl in MeOH (250 mL) under reflux for 1 h to yield **5**. After addition of 2N HCl the mixture was extracted 3 times with toluene (50 mL). The combined organic phases were dried and evaporated in vacuo. **5** was isolated as a yellow oil, yield 90%. ^{1}H NMR (DMSO-d_6) δ 10.5 (*s*, ^{1}H), 7.7 (*d*, ^{1}H, *J*=8.1 Hz), 6.9 (*d*, ^{1}H, *J*=1.6 Hz), 6.8 (*dd*, ^{1}H, *J*=1.6 Hz, *J*=8.1 Hz), 3.8 (*s*, ^{3}H), 3.7 (*s*, ^{2}H), 3.6 (*s*, ^{3}H); ^{13}C NMR (DMSO-d_6) δ 171.1, 169.2, 160.1, 142.6, 130.1, 120.9, 118.4, 111.9, 52.6, and 52.1; FD MS: *m/z* (%)=224.1 (100%); Anal. $C_{11}H_{12}O_5$ (calculated): C 59.17%, H 5.41% (C 58.93%, H 5.39%).

2.6. 4-Carboxymethyl-2-hydroxy-benzoic acid methyl ester 6

To a solution of **5** (4.0 g, 18 mmol) in MeOH (40 mL), 2N NaOH (18.9 mL) were added. After stirring for 1 h, the MeOH was removed in vacuo at 40°C. The aqueous phase was extracted 3 times with toluene (40 mL) and acidified to pH 2 with 2 N HCl. The aqueous phase was filtered and the precipitate washed with water to yield a yellowish solid, 64%. ^{1}H NMR (DMSO-d_6) δ 12.5 (*br*, ^{1}H), 10.4 (*s*, ^{1}H), 7.7 (*d*, ^{1}H, *J*=8.1 Hz), 6.8 (*d*, ^{1}H, *J*=1.4 Hz), 6.75 (*dd*, ^{1}H, *J*=1.4 Hz, *J*=8.1 Hz), 3.8 (*s*, ^{3}H), 3.5 (*s*, ^{2}H); ^{13}C NMR (DMSO-d_6) δ 172.1, 169.4, 160.1, 143.5, 130.0, 121.0, 118.4, 111.5, 50.6, and 40.8; FD MS: *m/z* (%)=210.8 (100%); Anal. $C_{10}H_{10}O_5$ (calculated): C 57.37%, H 4.94% (C 57.14%, H 4.80%).

2.7. (S)-2-Hydroxy-4-([3-methyl-1-(2-piperidin-1-yl-phenyl)-butylcarbamoyl]-methyl)-benzoic acid methyl ester 8

To a stirred solution of the amine **7** (0.246 g, 1 mmol) in toluene (30 mL), **6** (0.232 g, 1.1 mmol) was added at room temperature. After the mixture became clear, N,N′-dicyclohexylcarbodiimide (DCC) (0.272 g, 1.1 mmol) was added. The solution was stirred and monitored by TLC (*n*-hexane/ethylacetate 1:1) (~2 h). The reaction was filtered and the filtrate was evaporated in vacuo. The residue was purified via column chromatography (*n*-hexane/ethylacetate 1:1). The product was isolated as a white solid, yield 65%. ^{1}H NMR (DMSO-d_6) δ 10.4 (*s*, ^{1}H), 8.3 (*d*, ^{1}H), 7.6 (*d*, ^{1}H), 7.2 (*d*, ^{1}H), 7.0 (*dd*, ^{1}H), 7.0 (*d*, ^{1}H, *J*=7.5 Hz), 6.9 (*dd*, ^{1}H), 6.8 (*s*, ^{1}H), 6.7 (*d*, ^{1}H), 5.3 (*dt*, ^{1}H), 3.8 (*s*, ^{3}H), 3.4 (*d*, ^{2}H), 3.0 (m, ^{2}H), 2.5 (m, ^{2}H), 1.7- 1.1 (m, ^{9}H), 0.84 (*d*, ^{3}H), 0.82 (*d*, ^{3}H); ^{13}C NMR (DMSO-d_6) δ 169.5, 168.6, 160.2, 151.7, 145.3, 140.5, 129.9, 127.4, 126.2, 124.2, 120.7, 120.6, 117.9, 111.1, 54.2, 52.5, 46.7, 46.2, 42.6, 26.5, 25.1, 24.0, 23.3, 22.0; FD MS: *m/z* (%)=439.0 (100%); Anal. $C_{26}H_{34}N_2O_4$ (calculated): C 70.82%, H 7.77%, N 6.52% (C 71.21%, H 7.81%, N 6.39%).

2.8. (S)-2-(2-fluoroethoxy)-4-([3-methyl-1-(2-piperidin-1-yl-phenyl)-butylcarbamoyl]-methyl)-benzoic acid 9

To a stirred solution of **8** (860 mg, 1.96 mM) with K_2CO_3 (630 mg, 4.5 mmol) in acetone (5 mL), 1-bromo-2-fluoroethane (501 mg, 3 mmol) and NaI (10 mg, 66.7 μmol) were added. The reaction was refluxed for 20 h and monitored via TLC (*n*-hexane/ethylacetate 1:1), filtered and evaporated in vacuo. The residue was purified by column chromatography (*n*-hexane/ethylacetate 1:1) and yielded a white solid, 54%. ^{1}H NMR (acetone-d_6) δ 7.6 (*d*, ^{1}H), 7.5 (*d*, ^{1}H), 7.2 (*d*, ^{1}H), 7.1 (m, ^{2}H), 7.0 (*s*, ^{1}H), 6.9 (*dd*, ^{1}H), 6.9 (*d*, ^{1}H), 5.5 (*dt*, ^{1}H), 4.7 (*dt*, ^{2}H, *J*=3.9 Hz, *J*=48 Hz), 4.2 (*dt*, ^{2}H, *J*=3.9 Hz, *J*=29 Hz), 3.7 (*s*, ^{3}H), 3.5 (*s*, ^{2}H), 3.1 (m,

^{2}H), 2.6 (m, ^{2}H), 1.8 - 1.1 (m, ^{9}H), 0.9 (*d*, ^{3}H), 0.8 (*d*, ^{3}H); ^{13}C NMR (acetone-d_6) δ 168.2, 165.7, 157.7, 151.9, 142.4, 140.0, 130.9, 127.1, 126.0, 124.0, 121.4, 120.6, 119.3, 114.9, 82.5, 80.8, 68.5, 68.3, 53.9, 50.8, 46.5, 46.5, 42.8, 26.4, 24.9, 23.9, 22.4, and 21.4; ^{19}F NMR (acetone-d_6) −224.4 (*tt*, 1F, J=29 Hz, J=48 Hz); FD MS: *m/z* (%)=485.0 (100%); Anal. $C_{28}H_{37}FN_2O_4$ (calculated): C 69.25%, H 7.86%, N 5.80% (C 69.40%, H 7.70%, N 5.78%).

A solution of the intermediate (467 mg, 1 mM) in methanol (10 mL) was refluxed with 1N NaOH (2.1 mL, 2.1 mmol) and monitored via TLC (methanol/ethylacetate 1:9). The mixture was acidified with 1N HCl, cooled and filtered. The residue was washed with cold water and evaporated in vacuo. If further purification is required, column chromatography (methanol/EtOAc 1:9) can be performed to give a white solid, yield 93%. ^{1}H NMR (methanol-d_4) δ 8.4 (*d*, ^{1}H), 7.7 (*d*, ^{1}H), 7.2 (*d*, ^{1}H), 7.1 (m, ^{2}H), 7.0 (*dd*, ^{1}H), 6.97 (*s*, ^{1}H), 6.92 (*d*, ^{1}H), 5.5 (m, ^{1}H), 4.7 (*dt*, ^{2}H, J=3.9 Hz, J=48 Hz), 4.2 (*dt*, ^{2}H, J=3.9 Hz, J=28 Hz), 3.5 (*s*, ^{2}H), 3.0 (*m*, ^{2}H), 2.6 (m, ^{2}H), 1.8 - 1.2 (*m*, ^{9}H), 0.9 (*d*, ^{3}H), 0.89 (*d*, ^{3}H); ^{13}C NMR (methanol-d_4) δ 170.7, 167.9, 157.7, 151.8, 142.1, 138.8, 131.4, 127.3, 125.8, 124.3, 121.3, 120.6, 118.8, 113.9, 82.1, 80.4, 68.4, 68.2, 54.4, 46.6, 45.6, 42.3, 26.1, 24.9, 23.6, 21.9, and 21.0; ^{19}F NMR (methanol-d_4) δ −225.6 (*tt*, 1F, J=28 Hz, J=48 Hz); FD MS: *m/z* (%)=470.9 (100%); Anal. $C_{27}H_{35}FN_2O_4$ (calculated): C 68.99%, H 7.33%, N 6.04% (C 68.91%, H 7.50%, N 5.95%).

2.9. 2-[^{18}F]Fluoroethyltosylate

No-carrier-added (NCA) aqueous [^{18}F]fluoride (18000 MBq) prepared by the ^{18}O(p,n)^{18}F nuclear reaction on an enriched water (95%) target was added to a solution of 1 N K_2CO_3 (15 μL) and Kryptofix 2.2.2. (15 mg) in CH_3CN (800 μL). The water was removed by coevaporation to dryness with CH_3CN (2×1 mL) using a stream of nitrogen at 80°C.

To the dried Kryptofix 2.2.2./[^{18}F]fluoride complex (17000 MBq) in acetonitrile (1 mL) ethylenglycol-1,2-ditosylate (8–10 mg, 20–25 μmol) was added and heated under stirring in a sealed vial for 3 min. Purification of the crude product was accomplished using HPLC (acetonitrile/water, 50:50, flow rate: 5 mL/min r_t: 8 min). After diluting the HPLC fraction containing the 2-[^{18}F]fluoroethyltosylate with water (20 mL), the product was loaded on a C18-SepPac cartridge (Waters), dried with nitrogen gas and eluted with tempered (25–30°C) diethyl ether (2 mL) to yield the desired product 2-[^{18}F]fluoroethyltosylate with an activity of 8000 MBq. After evaporation of the diethyl ether in a stream of nitrogen, the 2-[^{18}F]fluoroethyltosylate was taken up in DMSO (150–200 μL).

2.10. (S)-2-(2-[^{18}F]fluoroethoxy)-4-([3-methyl-1-(2-piperidin-1-yl-phenyl)-butylcarbamoyl]-methyl)-benzoic acid ([^{18}F]repaglinide)

To **8** (3 mg, 6.8 μmol) dissolved in DMSO (250 μL) 1N NaOH solution was added (6.8 μL, 6.8 μmol) and the mixture was heated at 150°C for 2 min. A solution of 2-[^{18}F]fluoroethyltosylate (8000 MBq) in DMSO (150–200 μL) was added and stirred in a sealed reaction vessel at 150°C for 10 min. The product was purified with HPLC (acetonitrile/0.1 M acetic acid/sodium acetate buffer (pH=6) (8/2) (v/v), flow rate 4 mL/min, r_t: 10.2 min). After diluting the HPLC fraction containing the product with water (20 mL), it was loaded on a C18-SepPac cartridge (Waters), dried with nitrogen and eluted with methanol (1.5 mL) to yield (S)-2-(2-[^{18}F]fluoroethoxy)-4-([3-methyl-1-(2-piperidin-1-yl-phenyl)-butylcarbamoyl]-methyl)-benzoic acid methyl ester. 1N NaOH solution was added (100 μL) and stirred in a sealed reaction vessel at 80°C for 35 min. The mixture was neutralized with 1N HCl (100 μL). The product was purified with HPLC (acetonitrile/0.1 M acetic acid/sodium acetate buffer (pH=6) (8/2) (v/v), flow rate 4 mL/min, r_t: 4.5 min). After diluting the HPLC fraction containing the product with water (20 mL), it was loaded on a C18-SepPac cartridge (Waters), dried with nitrogen and eluted with warm ethanol (1 mL) to yield 1500 MBq of [^{18}F]repaglinide. HPLC analysis showed a radiochemical purity of >98%. Radio-TLC analysis confirmed the results (ethylacetate/methanol 9:1, R_f=0.7). The specific activity of [^{18}F]repaglinide was between 50 and 60 GBq/μmol as determined via a UV-calibration curve.

2.11. Competition binding experiments

To assess binding affinities of repaglinide and fluoroalkylated repaglinide **9** for human SUR1, [^{3}H]glibenclamide (0.3 nM) displacement assays were performed with membranes from COS-1 cells transiently expressing human SUR1.

2.12. Materials

[^{3}H]glibenclamide (specific activity 51 Ci mmol^{-1}) was purchased from NEN (Dreieich, Germany). Stock solutions of all drugs were prepared in KOH (50 mM) or dimethyl sulfoxide with a final solvent concentration in the media below 1%.

2.13. Binding assays

Transfections and membrane preparations were performed as described [19,20]. Briefly, COS-1 cells cultured in DMEM HG (10 mM glucose), supplemented with 10% fetal calf serum (FCS), were plated at a density of 5×10^5 cells per dish (94 mm) and allowed to attach overnight. 200 μg of pECE-human SUR1 complementary DNA (GenBank

Table 1
Insulin secretion in response to repaglinide or ^{19}F-derivative **9** from incubated islets. Ten islets were incubated for 60 min in the presence of (1 g/L glucose alone) or (1 g/L glucose + 0.10 μM repaglinide or ^{19}F-derivative **9**). Insulin secretion was also measured at 3 g/L glucose alone. Results were expressed as mean±SD. N = number of experiments.

	Insulin Release ng/Islet/1h (1 g/L glucose)	Insulin Release ng/Islet/1h (1 g/L glucose + RG)	Insulin Release ng/Islet/1h (3 g/L glucose)	Stimulation Index	Relative Stimulation Index
Positive Control	0.9 ± 0.2	. . .	3.7 ± 0.6	4.2 ± 0.5	100%
Repaglinide					
N = 8	0.7 ± 0.2	1.6 ± 0.2	. . .	2.4 ± 0.4	59%
^{19}F-derivative 9					
N = 8	0.8 ± 0.2	1.8 ± 0.4	. . .	2.3 ± 0.1	54%

NP_000343) were used to transfect 10 plates. For transfection the cells were incubated 4 h in a tris-buffered salt solution containing DNA (5–10 μg/mL) plus DEAE-dextran (1 mg/mL), 2 min in HEPES-buffered salt solution plus dimethyl sulfoxide (10%) and 4 h in DMEM-HG plus chloroquine (100 μM). Cells were then returned to DMEM-HG plus 10% FCS and were used 60–72 h post-transfection to prepare membranes as described [19]. To measure binding to membranes from COS-cells the resuspended fraction (final protein concentration 5–50 μg/mL) was incubated in "tris-buffer" (50 mM, pH 7.4) containing [^{3}H]glibenclamide (final concentration 0.3 nM, non-specific binding defined by 1 μM glibenclamide) and either fluorinated repaglinide **9**, repaglinide or unlabeled glibenclamide. Incubations were carried out for 1 h at room temperature and were terminated by rapid filtration through Whatman GF/B filters.

2.14. Data

Half-maximally inhibitory drug-concentrations (IC_{50} values) and Hill coefficients (n) were estimated by fitting the function $B=1/(1+([\text{drug}]/IC_{50})^n)$ to the data of each single displacement experiment. K_Ds were calculated from IC_{50} values as described [19]. Data shown as means ±S.E.M.

2.15. Insulin secretion experiments on rat islets

For testing the in vitro function of fluorinated repaglinide derivative **9** and repaglinide a standardized batch stimulation was performed according to a protocol established in our laboratory [21,22]. Adult rat islets were isolated by collagenase digestion and purified by a density gradient [23].

Briefly, lewis rats (Central Animal Facility, University of Mainz), 6–8 weeks old, body weight 250–270 g, were used as islet donors. Rats were anaesthetized by intraperitoneal pentobarbital administration (60 mg/kg); a midline abdominal incision was performed and the pancreas was exposed and injected via the pancreatic duct with Hanks' balanced salt solution (HBSS; Gibco BRL, Long Island, NY, USA) containing 1.7 mg/mL collagenase (Serva PanPlus, Heidelberg, Germany). After the death of the animals, the pancreatic tissue was surgically removed and incubated for 10 min at 37°C in the collagenase solution. Mechanical disruption of the digested pancreatic tissue was achieved by further incubation at 37°C for 10 min in collagenase solution, interrupted every 2 min by shaking for 30 s. The digestion process was stopped by addition of cooled HBSS plus 10% fetal calf serum (4°C). Islet purification was achieved using a discontinuous three-phase Ficoll density gradient (densities: 1.090, 1.077, and 1.040). Islets were cultured in RPMI medium (Biochrom KG, Berlin, Germany) at 37°C. The medium contained 5.1 mmol/L (1g/L) D-glucose, 25 mmol/L HEPES, 10% fetal calf serum (Greiner Laboratories, Frickenhausen, Germany), 0.2 g/L Glutamax (Gibco-BRL, Paisley, Scotland), penicillin, streptomycin (Gibco-BRL, Paisley, Scotland) and Ciprobay (Hoechst, Frankfurt, Germany).

For each sample ten islets were picked (equal in size and shape) in a culture-insert with a membrane of 12 μm pore size (Millicell PCF, Millipore, France) and incubated in a 24-well culture-plate (Falcon Multiwell, Becton Dickinson, USA). First, basal insulin secretion was tested by incubating the islets with normo-glycemic culture media (RPMI 1640+D-glucose 1 g/L+10% FCS) for 1 h at 37°C. After the culture period the media were collected and stored at −20°C. The inserts with islets were transferred to normoglycemic culture-medium containing 0.10 μM of repaglinide or ^{19}F-derivative **9** and incubated for a stimulation period of 1 h. As a positive control several inserts with islets were cultured in hyperglycemic culture-medium (RPMI 1640 D-glucose 15 mM+10% FCS) only. For negative control normo-glycemic culture-medium (RPMI 1640 D-glucose 5 mM+10% FCS) lacking repaglinide or compound **9** was used. The insulin content of each probe was quantified by a rat-insulin ELISA (Mercodia, Uppsala, Sweden). Insulin secretion was expressed as insulin release per islet/h. The stimulation index (Table 1) was calculated by dividing insulin output during stimulation (15 mM D-glucose or 5 mM D-glucose+test substance) by insulin secretion during basal incubation (5 mM D-glucose). The relative stimulation index was calculated by dividing insulin output during stimulation with 5 mM D-glucose+test substance by

insulin secretion during incubation with 15 mM D-glucose×100.

2.16. Results

The increase of insulin secretion after stimulation with compound **9** was determined to be 1.8±0.4 ng/islet/h and was in the same range as that of repaglinide (1.6±0.2 ng/islet/h) (Table 1). These results indicate that derivatisation of repaglinide does not significantly alter its insulin-stimulating properties.

2.17. Determination of log D values

log *D* values were determined according to the "OECD guideline for testing chemicals" applying the high-performance-liquid chromatography (HPLC) method [24,25].

2.18. In vivo evaluation of receptor binding

The radioactivity uptake of [^{18}F]repaglinide in different organs was assessed in Sprague-Dawley rats (Charles River Wiga, Sulzfeld, Germany; body weight 220–340 g; receiving a standard diet and water ad libitum). All experimentation had previously been approved by the regional animal ethics committee and was conducted according to German federal law. Animals were anaesthetized with pentobarbital (40 mg/kg, Narcoren®, Merial, Hallbergmoos, Germany). A catheter was placed in the carotic artery and the jugular vein, respectively. Animals breathed spontaneously through a tracheal tube during the entire experiment. [^{18}F]repaglinide was dissolved in isotonic saline containing 5% ethanol and was rapidly injected in the jugular vein with an activity of 15–25 MBq. At each of the set time points (5, 10, 20, 30, and 60 min post injection) 4 animals were taken out of the experiment and a small blood sample (~0.5 mL) obtained from the arterial catheter. The respective animals were then sacrificed and samples from different organs (heart, lung, spleen, liver, pancreas, kidneys, and brain) were taken. The tissues were weighted and subsequently dissolved in 4.0 mL KOH (4N) at 75°C for 30 min. The samples were then measured in a γ-counter. In the displacement study 4 animals were injected with 25–30 MBq [^{18}F]repaglinide. 10 minutes p.i. repaglinide (0.1 mg/kg body weight) dissolved in isotonic saline containing 5% ethanol were injected and the animals were sacrificed 20 minutes p.i. of the [^{18}F]repaglinide. The corresponding organs were removed, weighted, dissolved in KOH and measured in a γ-counter.

3. Results and discussion

The syntheses of the ^{19}F-standard compound **9** for evaluating the biological activity and the labeling precursor **8** for the labeling reaction with 2-[^{18}F]fluoroethyltosylate started from 2-hydroxy-4-methyl benzoic acid **1**. The synthetic strategy of Grell et al. for the syntheses of repaglinide and related hypoglycemic benzoic acid derivatives was applied in a modified form (Fig. 1) [18]. Esterfication with methanol and sulfuric acid yielded the corresponding methyl ester **2**. After side chain bromination of **2** with NBS and azo-bis-isobutyronitril (AIBN) as a radical starter, the bromo compound **3** was reacted with NaCN in water using N-benzyltributyl ammonium chloride as a phase catalyst to yield **4**. Hydrolysis of the nitrile moiety was conducted by continuous introduction of gaseous HCl into a methanolic solution. The bis-methyl ester **5** was obtained in high yields and could be selectively cleaved with 2.1 equivalents NaOH to obtain the mono-ester 4-carboxymethyl-2-hydroxy benzoic acid **6**. The mono-ester **6** was coupled with (S)-3-methyl-1-[2-(1-piperidinyl)phenyl)-butylamine **7** [18] applying DCC as a coupling agent leading to the final labeling precursor (S)-2-hydroxy-4-((3-methyl-1-(2-piperidin-1-yl-phenyl)-butylcarbamoyl)-methyl) benzoic acid **8**. The following reaction with 1-bromo-2-fluoroethane in acetone led to the methylester protected non-radioactive standard com-

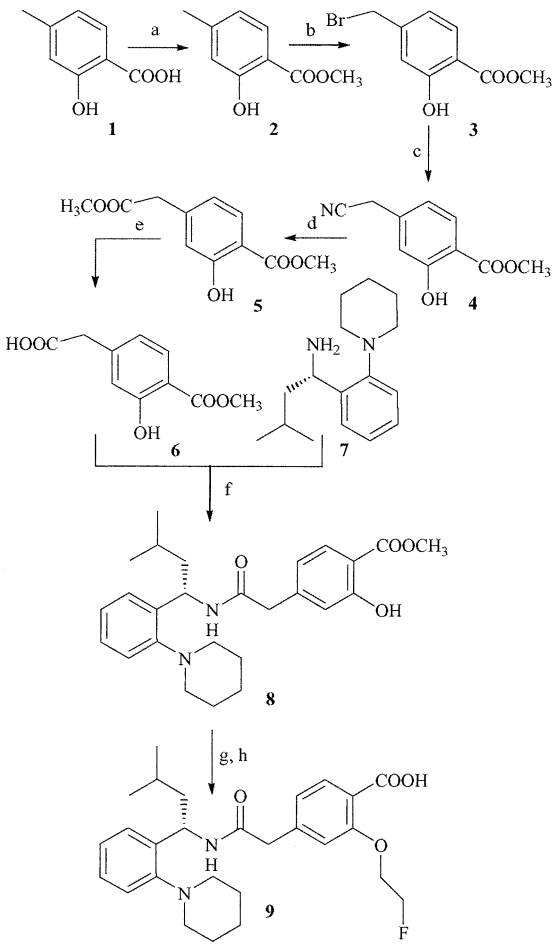

Fig. 1. Syntheses of the labeling precursor and the [^{19}F]repaglinide standard compound **9** for in vitro evaluation. (a) CH_3OH/H_2SO_4; (b) NBS/AIBN/CCl_4; (c) NaCN/N-benzyltributylN^+Cl^-/H_2O; (*d*) HCl/CH_3OH; (e) NaOH/MeOH; (f) DCC/toluene; (g) 1-bromo-2-fluoroethane/acetone; and (h) $NaOH/CH_3OH$.

pound. Interestingly, no formation of the 2-fluoroethyl-ester occurred without adding a catalytic amount of NaI. Even with longer reaction times, no product could be detected. Cleavage of the ester moiety was achieved using 2N NaOH yielding the final [^{19}F]repaglinide derivative **9** for in vitro evaluation purposes.

3.1. Radioactive labeling

The radioactive labeling of **8** was conducted applying the secondary labeling precursor 2-[^{18}F]fluoroethyltosylate first introduced by Block et al. [26] with an overall radiochemical yield of 20% (decay corrected to the end of bombardment (EOB)). No-carrier-added (NCA) aqueous [^{18}F]fluoride prepared by the ^{18}O(p,n)^{18}F nuclear reaction on an enriched water target (95+% ^{18}O) was dried via co-distillation with acetonitrile applying a commonly used procedure [27]. 2-[^{18}F]Fluoroethyltosylate was synthesized via nucleophilic reaction of glycol-1,2-ditosylate with K[^{18}F]/kryptofix 2.2.2 and subsequently reacted with **8** in DMSO at 150°C for 10 min. After injection of the crude reaction mixture into an HPLC system the product peak was collected, diluted with water and the radioactive product was fixed onto a solid phase cartridge, dried with nitrogen and finally eluted with methanol. To the methanol phase containing the methyl-ester protected [^{18}F]repaglinide, 1 N NaOH was added and heated to 80°C for 35 min. This relatively long deprotection step is necessary to avoid unwanted decomposition of [^{18}F]repaglinide at higher temperatures which becomes predominant when raising the temperature up to 100°C leading to a dramatically decreased radiochemical yield (RCY). Probably the amide is cleaved under too harsh conditions. The solution was acidified with 2 N HCl and purified via HPLC to yield the final [^{18}F]repaglinide in an overall RCY of 20%.

3.2. Biochemistry

For testing the in vitro behavior of the non-radioactive fluorinated repaglinide (compound **9**), binding assays as well as insulin secretion experiments were performed to clarify whether the exchange of an ethoxy-group to a 2-fluoroethoxy-group affects biological activity. Competition binding experiments were performed to assess the affinity of repaglinide and compound **9** for binding to human SUR1 [19,20].

Unlabeled repaglinide and its fluorinated derivative induced complete monophasic inhibition curves with Hill coefficients close to 1 yielding dissociation constants (K_Ds) of 50.3 and 134 nM and IC_{50} values of 99.8 and 265.0 nM, respectively. The slight decrease in affinity of compound **9** may be due to its slightly higher hydrophilicity. The log D value of 2.39 for compound **9** in comparison to 2.52 for repaglinide supports this assumption. Insulin secretion experiments using a standardized batch stimulation on isolated rat islets show that the stimulatory effect of 100 nM for compound **9** is comparable to that of repaglinide (Table 1). Thus, derivatization of repaglinide does not significantly alter its insulin-stimulating properties.

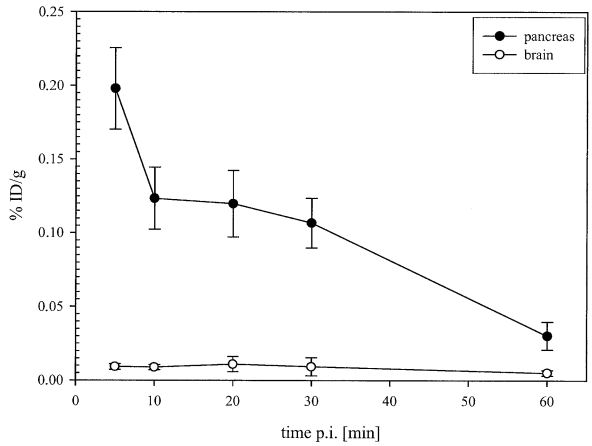

Fig. 2. Time-activity curves for comparison of radioactivity uptake in rats (n=4) in SUR1 bearing tissue such as pancreas and brain.

Preliminary in vivo biodistribution was assessed in Sprague Dawley rats. Animals were sacrificed at different time points (5, 10, 20, 30, and 60 min p.i.) and multiple organ samples were taken. [^{18}F]Repaglinide organ uptake in % of injected dose/gram (% ID/g) of the pancreas, lung, brain, liver, kidneys, spleen, heart, and blood were determined (Figs. 2 and 3). From 10 to 30 min p.i. pancreatic tissue displayed a stable accumulation of ≈0.12% of the injected [^{18}F]repaglinide dose. As 50% of the radioactive tracer could be displaced by additional injection of 0.1 mg/kg unlabeled repaglinide at t=10 min p.i., this accumulation might indicate specific binding to SUR1.

4. Conclusion

In summary, we synthesized the fluorinated repaglinide derivative (S)-2-(2-[^{18}F]fluoroethoxy)-4-((3-methyl-1-(2-piperidin-1-yl-phenyl)-butyl-carbamoyl)-methyl)-benzoic acid ([^{18}F]repaglinide) as the first compound for investigating the in vivo behavior of this novel class of antidiabetics non-invasively with PET. We synthesized both the labeling precursor **8** and the non-radioactive standard compound **9** in sufficient chemical and radiochemical yields. Radioactive labeling was performed in an overall RCY of 20% applying 2-[^{18}F]fluoroethyltosylate to yield [^{18}F]repaglinide with high specific activity as a sterile injectable saline solution. The corresponding non-radioactive ^{19}F-standard compound **9** displays binding and insulin secretion properties similar to repaglinide. Preliminary in vivo data suggest a specific binding of [^{18}F]repaglinide to SUR1. Further in vivo biodistribution studies and a first human PET scan are under investigation.

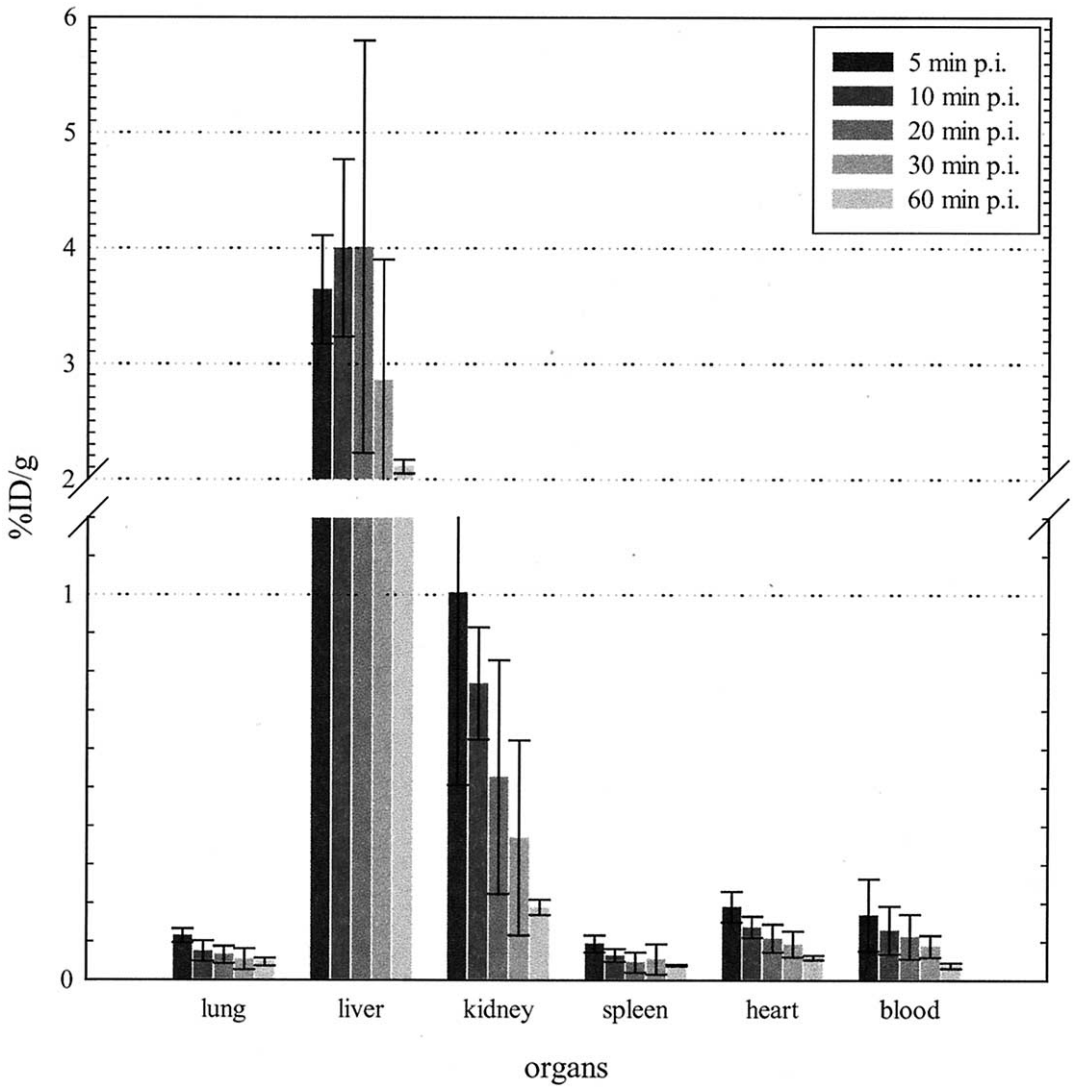

Fig. 3. In vivo biodistribution data of radioactivity in different organs in rats ($n=4$) at several time points after injection of [^{18}F]repaglinide.

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