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Synthesis and in vitro evaluation of (*S*)-2-([^{11}C]methoxy)-4-[3-methyl-1-(2-piperidine-1-yl-phenyl)-butyl-carbamoyl]-benzoic acid ([^{11}C]methoxy-repaglinide): a potential β-cell imaging agent

Björn Wängler,[a] Carmen Beck,[a] Chyng Yann Shiue,[b] Stephan Schneider,[c] Christina Schwanstecher,[d] Mathias Schwanstecher,[d] Peter Johannes Feilen,[c] Abass Alavi,[b] Frank Rösch[a] and Ralf Schirrmacher[a,*]

[a]*Institute of Nuclear Chemistry, University of Mainz, Mainz, Germany*
[b]*Department of Radiology, University of Pennsylvania, PA, USA*
[c]*Division of Endocrinology and Metabolism, I. Medical Department, University of Mainz, Mainz, Germany*
[d]*Institute of Pharmacology and Toxicology, University of Braunschweig, Braunschweig, Germany*

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Abstract—The ^{11}C-labeled sulfonylurea receptor 1 (SUR1) ligand (*S*)-2-([^{11}C]methoxy)-4-[3-methyl-1-(2-piperidine-1-yl-phenyl)-butyl-carbamoyl]-benzoic acid ([^{11}C]methoxy-repaglinide) was synthesized in an overall radiochemical yield of 35% after 55 min with a radiochemical purity higher than 99%. This compound is considered for the noninvasive investigation of the SUR1 receptor status of pancreatic β-cells by positron emission tomography (PET) in the context of type 1 and type 2 diabetes. The specific activity was 40–70 GBq/μmol. In vitro testing of the nonradioactive methoxy-repaglinide was performed to characterize the affinity for binding to the human SUR1 isoform. Methoxy-repaglinide induced a complete monophasic inhibition curve with a Hill coefficient close to 1 (1.03) yielding a dissociation constant (K_D) of 83 nM and an IC_{50} of 163 nM. Insulin secretion experiments on isolated rat islets were performed to prove biological activity, which was determined to be in the same range as that of original repaglinide.

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, 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]

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. Repaglinide is a novel fast acting prandial glucose regulator with a short plasma half-life (<1 h).[5–7] Different from the sulfonylurea class, 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

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* Corresponding author. Tel.: +49-61313925371; fax: +49-61313924510; e-mail: schirrma@uni-mainz.de

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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.[9–11] Recently, enantiomerically pure [^{14}C]repaglinide was used to obtain in vivo data about its metabolism and way of excretion.[12] A detriment of this study was the low specific activity (2–50 Ci/mmol) of the radiotracer and additionally the use of carbon-14 whose radiation characteristics (short range β^- particle, half-life $t_{1/2}$ = 5730 a) preclude a noninvasive investigation. Noninvasive investigations may become available by using a radioactive analogue with high specific activity and labeled with a positron emitting radionuclide, for example, ^{11}C or ^{18}F. Positron emission tomography (PET) is a promising imaging technique to quantitatively assess biodistribution of radiolabeled pharmaceuticals noninvasively in humans and animals in vivo. This method is pre-destined to quantify and visualize the receptor status of diverse receptor systems. Radiolabeled sulfonylureas such as ^{18}F-labeled glibenclamide and tolbutamide derivatives have been described[13,14] and reviewed recently.[15] These compounds have been proven to be unsuitable for β-cell quantification by Shiue and co-workers.[16] Recently Sweet et al. performed a systematic screening of potential β-cell imaging agents such as glibenclamide, tolbutamide, serotonin, L-DOPA, dopamin, nicotinamide, fluorodeoxyglucose, and fluorodithizone and concluded that none of these compounds were likely candidates providing sufficient specificity for quantification with PET.[17] Most recently our group investigated a promising ^{18}F-labeled derivative of repaglinide whose accumulation in rat pancreas could be displaced by repaglinide, indicating specific binding of that radiolabeled compound. The pancreatic tissue displayed a stable and fast accumulation of radioactivity from 10 to 30 min p.i.[18]

Unfortunately, the K_D value of the fluorine-labeled repaglinide derivative was 2.8-fold decreased versus repaglinide (142 ± 6 and 50 ± 4 nM, respectively; n = 5 each; $P < 0.05$) indicating that ^{18}F-derivatization leads to a decrease of the binding affinity (Fig. 1). In order to obtain a compound with higher affinity than the ^{18}F-derivative we synthesized the [^{11}C]methoxy derivative **3** of repaglinide. Derivatization in terms of methylation is supposed to induce less alteration in affinity and pharmacology as compared to fluoroalkylation. With regard to the ^{11}C-labeling, introduction of a ^{11}C-methyl group is simpler to handle than a ^{11}C-ethylation, which would provide the structurally unchanged ^{11}C-repaglinide. Fortunately, the analogous nonradioactive methoxy derivative **2** revealed an affinity significantly higher (83 ± 5 nM; n = 5) than the fluorinated derivative ($p < 0.05$) and similar to repaglinide itself (Fig. 1). The higher affinity (K_D 83 nM vs 142 nM) suggests the ^{11}C-repaglinide derivative to be more suitable for receptor visualization than the ^{18}F-derivative. The shorter physical half-life of ^{11}C ($t_{1/2}$ = 20 min) in comparison to ^{18}F ($t_{1/2}$ = 109 min) does not appear as a drawback since repaglinide rapidly accumulates within the pancreas reaching its maximum 10–30 min p.i.

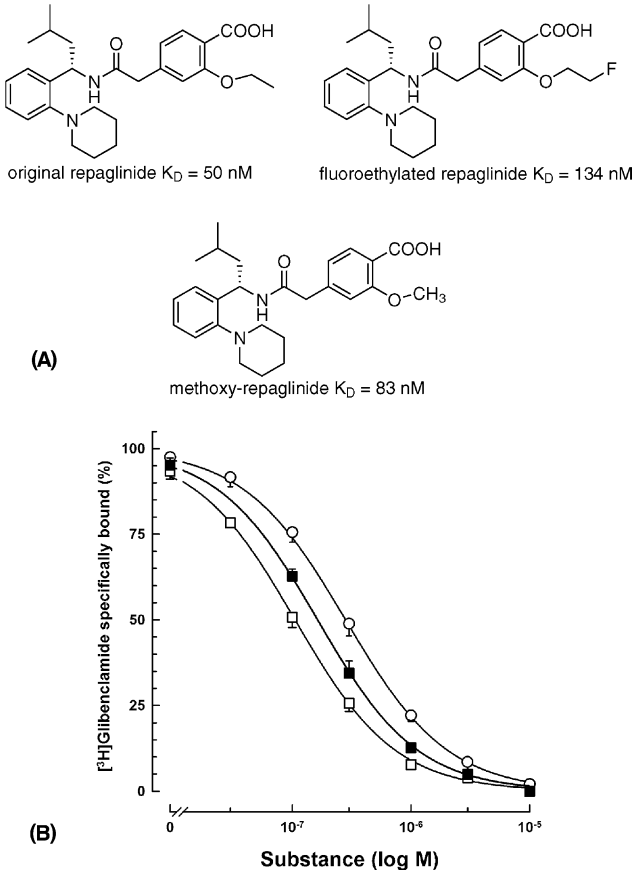

Figure 1. Binding affinities of repaglinide and the novel repaglinide derivatives for human SUR1. **A**: Structures of original repaglinide, fluoroethylated repaglinide, and methoxy-repaglinide. **B**: [^{3}H]glibenclamide (0.3 nM) displacement assays were done with membranes from COS-1 cells transiently expressing human SUR1. All incubations were performed in Tris–buffer (50 mM, pH 7.4) containing displacing drugs as indicated. The IC_{50} values (half-maximally inhibitory concentrations) and Hill coefficients are: 106 ± 7 nM, 1.03 (repaglinide, □); 163 ± 9 nM, 1.03 (methoxy-repaglinide, ■); 281 ± 11 nM, 1.01 (fluoroethylated repaglinide, ○). In parallel controls displacement by unlabelled glibenclamide was assessed (IC_{50} = 0.61 ± 0.03 nM, Hill coefficient = 1.00, data not shown). Results shown as mean ± SEM (n = 5). K_Ds were calculated from IC_{50} values as described.[23]

A ^{11}C-labeled repaglinide derivative with high specific activity might thus become a valuable tracer probe for the visualization and quantification of human pancreatic β-cell mass in vivo.

1. Chemistry

The nonradioactive 'standard' compound (*S*)-2-(methoxy)-4-[3-methyl-1-(2-piperidine-1-yl-phenyl)-butyl-carbamoyl]-benzoic acid (methoxy-repaglinide) (**2**),[19] required for in vitro evaluation and analytical purposes, was synthesized from the precursor (*S*)-2-hydroxy-4-([3-methyl-1-(2-piperidin-1-yl-phenyl)-butylcarbamoyl]-methyl)-benzoic acid methyl ester (**1**) and methyliodide in refluxing acetone with K_2CO_3 as a base following a similar procedure as described previously (Fig. 2).[18] Finally, the methylester moiety was cleaved with NaOH (1 N) in

refluxing methanol. Enantiomeric purity was proven by chiral high-performance liquid chromatography (HPLC) (Fig. 2).

For radioactive synthesis, the ^{11}C isotope was produced via the $^{14}N(p,\alpha)^{11}C$ nuclear reaction. The [^{11}C]methyliodide was synthesized with an automated synthesis module (PETtrace MeI MicroLab, GE Medical Systems) within 12 min. To 1 (2.1 mg, 4.8 μmol) dissolved in 250–350 μL DMF, 1 N NaOH solution was added (4.8 μL, 4.8 μmol) and the mixture was heated at 100 °C for 1 min. A solution of [^{11}C]methyliodide (1.9–2.2 GBq) in DMF (350–450 μL) was added and stirred in a sealed reaction vessel at 100 °C for 2.5 min. The intermediate product was purified with HPLC (acetonitrile/0.1 M acetic acid/Na acetate buffer (pH = 5) 8:2, flow rate 4 mL/min, t_r = 10.5 min).[20] After diluting the HPLC fraction containing the product with 15 mL water, it was loaded on a C18-SepPac cartridge (Waters) during 7 min, washed with 2 mL water, dried with nitrogen for 1 min and eluted with 1.5 mL ethanol to yield the intermediate (*S*)-2-([^{11}C]methoxy)-4-[3-methyl-1-(2-piperidine-1-yl-phenyl)-butyl-carbamoyl]-benzoic acid methyl ester. 1 N NaOH solution was added (100 μL, 100 μmol) and stirred in a sealed reaction vessel at 100 °C for 10 min. The mixture was neutralized with 1 M HCl (100 μL, 100 μmol). The product was purified by diluting with 15 mL water, loading on a C18-SepPac cartridge (Waters), drying with nitrogen for 1 min and eluting with 1 mL warm ethanol to yield 220–250 MBq of the product. Dilution with physiological saline and sterile filtration gave the injectable solution for in vivo experiments. The amount of physiological saline was adjusted so that 100 MBq of the product were dissolved in 2 mL solution and the final ethanol concentration was below 5%. HPLC analysis showed a radiochemical purity of >99%. The novel radiotracer was obtained in an overall radiochemical yield of 35% with a specific activity of 40–70 GBq/μmol, determined via a UV calibration curve. Enantiomeric purity was determined according to Grell et al.[21] to ensure that the ester hydrolysis does not affect the enantiomeric purity. The ^{11}C-methylation is obviously much easier to perform than the previously published ^{18}F-fluoroalkylation probably due the better leaving group properties of iodine. Furthermore, a ^{11}C-methylation is sterically less challenging than a ^{18}F-fluoroethylation, which makes the synthesis of **3** easier to perform.

2. In vitro evaluation of the nonradioactive standard compound 2 by binding- and insulin secretion experiments

To assess binding affinities of repaglinide and methoxy-repaglinide (**2**) for human SUR1, [^3H]glibenclamide (0.3 nM)[22] displacement assays were performed with membranes from COS-1 cells transiently expressing human SUR1. Transfections and membrane preparations were performed as described.[23,24] 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. Two hundred micrograms of pECE-human

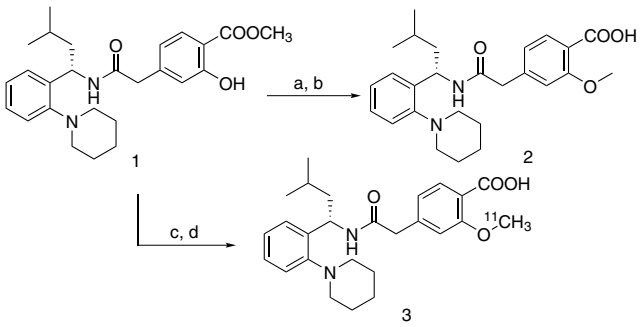

Figure 2. Synthesis of the non-radioactive standard compound **2** and radioactive labeling of **3**; Reagents and conditions: (a) methyliodide (1.2 equiv), NaI, acetone, 12 h; (b) NaOH (1 N) 1 equiv, methanol; (c) [^{11}C]methyliodide, NaOH (1 N), DMF, 100 °C, 2.5 min; (d) NaOH (1 N), 100 °C, 10 min.

SUR1 complementary DNA (GenBank 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.[23] 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 [^3H]glibenclamide (final concentration 0.3 nM, nonspecific binding defined by 1 μM glibenclamide) and either methoxy-repaglinide **2**, 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.

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.[23] Data shown as means ± S.E.M. Unlabeled repaglinide, its methoxy derivative **2** and fluoroethyl derivative induced complete monophasic inhibition curves with Hill coefficients close to 1 yielding dissociation constants (K_D) of 53, 83, and 142, respectively (Fig. 1).

For testing the in vitro function of **2**, a standardized batch stimulation was performed according to a protocol established in our laboratory.[14,25] Adult rat islets were isolated by collagenase digestion and purified by a density gradient. Briefly, Sprague Dawley rats (Central Animal Facility, University of Mainz), 6–8 weeks old and with a body weight of 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) containing 1.7 mg/mL collagenase (Serva PanPlus, Heidelberg, Germany). After the death of the animals, the pancreatic tissue was surgically removed

Table 1. Insulin stimulating capacity of repaglinide and its methoxy-derivative **2**

	Insulin release[a]	Insuline release[b]	Insuline release[c]	Stimulation index [%]	Relative stimulation index [%]
Positive control	2.3 ± 1.0	—	7.5 ± 3.3	3.2 ± 0.7	100
Repaglinide $N = 8$	2.1 ± 0.7	4.0 ± 1.4	—	1.9 ± 0.4	63
(**2**) $N = 8$	2.0 ± 0.6	4.0 ± 1.5	—	2.0 ± 0.4	64

Data are given as mean ± SE.
[a] ng/islet/1 h (5 mM glucose).
[b] ng/islet/1 h (5 mM glucose + 0.1 μM repaglinide or (**2**)).
[c] ng/islet/1 h (15 mM glucose).

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 the 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 (1 g/L) D-glucose, 25 mmol/L HEPES, 10% fetal calf serum (Greiner Laboratories, Frickenhausen, Germany), 0.2 g/L Glutamax (GibcoBRL, Paisley, Scotland) and antibiotics (100 units/mL penicillin, 100 μg/mL streptomycin; GibcoBRL, Paisley, Scotland and 10 μg/mL Ciprobay; Bayer, Leverkusen, Germany). For each sample, 10 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 normo-glycemic culture-medium containing 0.10 μM of repaglinide or its methoxy derivative **2** 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 its methoxy derivative **2** 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. The increase of insulin secretion after stimulation with compound **2** was determined to be 2.0 ± 0.4 ng/islet/h and was in the same range as that of repaglinide (1.9 ± 0.4 ng/islet/h) (Table 1). These results indicate that the described derivatization of repaglinide does not alter its insulin-stimulating properties.

3. Conclusion

[^{11}C]Methoxy-repaglinide (**3**), a derivative of the SUR specific ligand repaglinide was synthesized as an enantiomerically pure compound with an overall radiochemical yield of 35% after 55 min with a radiochemical purity >99% ($n = 8$). The radioactive synthesis is easy to perform and the simple labeling with [^{11}C]methyliodide is an advantage in comparison to a possible ^{11}C- ethylation for obtaining the structurally unchanged repaglinide. In comparison to ^{18}F-labeled repaglinide, the synthesis is more comfortable due to the availability of [^{11}C]methyliodide via a commercially available synthesis module. In vitro evaluation studies of the non-radioactive methoxy analog **2** showed that this compound binds with high affinity to the human SUR1 receptor of the pancreas and biological activity was totally retained as proven by insulin secretion experiments.

Thus, further evaluations with [^{11}C]methoxy-repaglinide are planned to elucidate its use for scientific and clinical studies using quantitative PET, although the imaging of β-cell loss is problematic due to the fainting imaging signal with progressing disease.

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19. Compound **2** was purified with column chromatography (Si-60): solvent ethylacetate/*n*-hexane; [$R_f = 0.55$]. ^{1}H NMR (400 MHz, MeOH-d_4): δ 7.5 (d, 1H, $J = 7.8$ Hz), 7.2 (d, 1H, $J = 7.8$ Hz), 7.1 (m, 2H), 7.0 (t, 1H), 6.9 (s, 1H), 6.8 (d, 1H), 5.5 (m, 1H), 3.7 (s, 3H), 3.5 (s, 2H), 3.0 (m, 2H), 2.6 (m, 2H), 1.8–1.1 (m, 9H), 0.9 (m, 6H); ^{13}C NMR (MeOH-d_4): δ 171.9, 171.0, 157.5, 151.9, 139.5, 139.1, 129.7, 127.1, 125.7, 124.0, 123.7, 120.6, 120.3, 111.7, 54.6, 46.0, 42.5, 33.1, 26.3, 24.9, 24.5, 23.8, 22.0, 21.1; FD MS: *m/z* (%) = 439.3 (100%).
20. HPLC-column: quality control: (a) Phenomenex Luna 5 μm C-18 (4.6 × 250), flow: 1 mL/min, solvent: acetonitrile/0.1 M acetic acid/Na acetate buffer (pH = 5) 8:2, t_r 1 = 13.0 min, t_r 2 = 3.9 min, t_r (intermediate) = 9.6 min; (b) preparative purification: Phenomenex Luna 5 μm C-18 (10 × 250), flow: 4 mL/min, solvent: acetonitrile/0.1 M acetic acid/Na acetate buffer (pH = 5) 8:2, t_r 1 = 16.1 min, t_r 2 = 4.6 min, t_r (intermediate) = 10.5 min.
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