ABSTRACT

Purpose: Despite excellent radionuclide characteristics, no 18F-labeled peptides are available for quantitative peptide receptor mapping using positron emission tomography (PET) so far, mainly due to time-consuming multistep radiosyntheses with limited overall yields. A newly developed two-step chemoselective conjugation method allows rapid and high-yield 18F-fluorination of peptides via oxime formation and was applied for the synthesis of new 18F-labeled carbohydrated Tyr3-octreotate (TOCA) analogs with optimized pharmacokinetics suitable for clinical routine somatostatin receptor (sst) imaging.

Experimental Design: 18F-labeled glucose (Gluc-S-) and cellubiose (Cel-S-) derivatives of aminooxy-functionalized TOCA were synthesized via oxime formation with 4-18F-fluorobenzaldehyde ([18F]FBOA-peptides). Both the in vitro internalization profile of Gluc-S-Dpr([18F]FBOA)TOCA and Cel-S-Dpr([18F]FBOA)TOCA in hss2t-expressing Chinese hamster ovary cells (dual tracer protocol) and their biodistribution in AR42J tumor-bearing mice were investigated and compared with two 18F-fluoropropionylated ([18F]FP) analogs, Gluc-Lys([18F]FP)TOCA and Gluc-S-Dpr([18F]FP)TOCA.

Results: In contrast to [18F]FP-labeling (3 h), chemoselective [18F]FBOA-formation (50 min) afforded the respective radiopeptides in high yields (65–85%). In vitro, Gluc-S-Dpr([18F]FBOA)TOCA and Cel-S-Dpr([18F]FBOA)-TOCA showed high internalization (139 ± 2 and 163 ± 8 of the reference [125I]Tyr3-octreotide, respectively), which was reflected by high tumor accumulation in vivo [21.8 ± 1.4 and 24.0 ± 2.5% of injected dose/g (1 h), respectively]. However, only Cel-S-Dpr([18F]FBOA)TOCA and Gluc-S-Dpr([18F]FP)TOCA (tumor: 15.1 ± 1.5% of injected dose/g) with its very low accumulation in all of the nontarget organs showed improved tumor:organ ratios compared with Gluc-Lys([18F]FP)TOCA, ForCel-S-Dpr([18F]FBOA)TOCA, tumor:organ ratios (1 h) were 42:1, 27:1, 15:1, 3:1, and 208:1 for blood, liver, intestine, kidney, and muscle, respectively.

Conclusion: Due to the fast and high-yield chemoselective radiofluorination strategy and to its excellent pharmacokinetics, Cel-S-Dpr([18F]FBOA)TOCA represents the first tracer suitable for routine clinical application in PET somatostatin receptor imaging.

INTRODUCTION

The success of the 111In-labeled octreotide analog [111In]-octreoscan for in vivo detection of somatostatin receptor overexpressing human neoplasms using single photon emission computed tomography (SPECT) is still unchallenged. Compared with single photon emission computed tomography; however, positron emission tomography (PET) represents a superior detection technique with respect to sensitivity, resolution, and quantification. To be able to benefit from these physical advantages, intense research has been directed toward the development of suitable octreotide analogs labeled with positron (β+) emitting radionuclides during the last decade.

Due to the ease of radiometallation, a variety of chelator-coupled octreotide derivatives have been labeled with positron-emitting radionuclides such as 86Y (1–3), 64Cu (4–6), and 66/68Ga (7–10). Initial patient PET studies using 64Cu-triethylenetetra-amine-octreotide as well as [66Ga]DOTA0-d-Phe1-Tyr3-octreotide ([66Ga]DOTATOC) and [68Ga]DOTATOC have been performed recently. Especially the latter two showed excellent imaging characteristics and high tumor:background ratios. [86Y]DOTATOC was successfully applied for dose estimation before peptide receptor-mediated radionuclide therapy (PRRT) using [68Y]DOTATOC (3). Optimization of the chelator moiety with respect to complex stability, radioligand pharmacokinetics, and receptor affinity led to additional very promising TOC analogs for PET as well as PRRT applications (11). None of the radiometals 86Y, 64Cu, and 66Ga, however, has optimal radionuclide characteristics for routine PET imaging purposes. They suffer from less than optimal half-lives (86Y: 14.7 h, 64Cu: 12.7 h, and 66Ga: 9.49 h), low β−-percentage branching (86Y: 33%, 64Cu: 18%, and 66Ga: 57%), high β−-energy (86Y: 1.3 MeV, and 66Ga: 1.7 MeV), as well as coemission of a substantial amount of γ-radiation [66Ga: also β−coemission].

First 18F-Labeled Tracer Suitable for Routine Clinical Imaging of sst Receptor-Expressing Tumors Using Positron Emission Tomography

Margret Schottelius,1 Thorsten Poethko,1 Michael Herz,2 Jean-Claude Reubi,2 Horst Kessler,3 Markus Schwaiger,1 and Hans-Jürgen Wester1

1 Nuklearmedizinische Klinik und Poliklinik, Klinikum rechts der Isar, Technische Universität München, München, Germany; 2 Institute of Pathology, University of Berne, Berne, Switzerland; and 3 Institut für Organische Chemie und Biochemie, Technische Universität München, Garching, Germany

Vol. 10, 3593–3606, June 1, 2004
Clinical Cancer Research 3593
all of which results in increased radiation doses for the patient. Although $^{68}$Ga (68 min; 1.8 MeV) already represents a promising radionuclide for PET and, furthermore, is easily accessible by the availability of $^{68}$Ge generators, $^{18}$F with its half-life of 120 min and low $\beta^+$-energy (0.64 MeV) represents the ideal radionuclide for routine PET imaging (12, 13). To exploit these characteristics for PET sst imaging, $^{18}$F-labeled octreotide analogs have been prepared using $[^{18}\text{F}]$fluoroacylation reactions. The first two analogs investigated, 2-$[^{18}\text{F}]$fluoropropionyl-$\text{D-Phe}^1$-octreotide (1, 14) and 4-$[^{18}\text{F}]$fluorobenzoyl-$\text{D-Phe}^1$-octreotide (15) showed low tumor uptake, low tumor retention, and, due to their high lipophilicity, unfavorable biokinetics.

It has been shown recently that sugar conjugation is a powerful method to improve radioligand pharmacokinetics. Carbohydration leads to reduced lipophilicity of small radiolabelled peptides and, thus, to a dramatic reduction of hepatobiliary in favor of renal excretion (16–20). Furthermore, it has been demonstrated that depending on the carbohydrate used tumor uptake of radioiodinated TOC and Tyr$^3$-octreotide (TOCA) may be substantially increased by glycosylation (19–21). On the basis of these findings, an $^{18}$F-labeled sugar analog of TOCA, N$^\omega$-(1-deoxy-$\alpha$-fructosyl)-N$^\omega$-(2-$[^{18}\text{F}]$fluoropropionyl)-Lys$^5$-Tyr$^3$-octreotide (Gluc-Lys($^{[18}\text{F}]$FP)-TOCA; Ref. 22) has been developed. To allow both glycosylation and prosthetic group labeling, both of which require a free amino functionality in the peptide, Lys$^5$ was introduced as a trifunctional linker (Fig. 1). In AR42J tumor-bearing nude mice, Gluc-Lys($^{[18}\text{F}]$FP)-TOCA showed very low hepatic and intestinal uptake, renal excretion, and high tumor accumulation. Initial patient PET studies performed with this radioligand demonstrated excellent tumor:background ratios even at time points $\ll 1$ h (22).

One significant drawback of Gluc-Lys($^{[18}\text{F}]$FP)-TOCA, especially with respect to clinical routine application, is generally encountered in $^{18}$F-labeling of small bioactive peptides via prosthetic groups (1, 13, 14, 12–26): the time consuming, multistep radiosynthesis of the $[^{18}\text{F}]$fluorinated precursor used for prosthetic group labeling, in this case 4-nitrophenyl 2-$[^{18}\text{F}]$fluoropropionate for $[^{18}\text{F}]$fluoroacylation. Furthermore, the presence of a second amino functionality (Lys$^5$) in the receptor-binding sequence of TOCA requires the use of a protected precursor peptide, which, in turn, necessitates an additional deprotection and purification step after the $[^{18}\text{F}]$fluoroacylation. This leads to an overall preparation time of Gluc-Lys($^{[18}\text{F}]$FP)-TOCA of $\sim$3 h and, thus, to comparably low radiochemical yields.

To overcome these disadvantages—in this particular case as well as concerning $^{18}$F-labeling of peptides and proteins in general—a new strategy ideally allowing both one-step, high-yield synthesis of an $^{18}$F-labeled prosthetic group with high stability against in vivo defluorination and fast, one-step, chemoselective conjugation with unprotected peptides under mild conditions, preferably in aqueous media, was needed. The chemoselective formation of an oxime bond between a radiohalogenated ketone or aldehyde, e.g., 4-$[^{18}\text{F}]$fluorobenzaldehyde, and a peptide functionalized with an aminoxy-functionality fulfills both requirements. This methodology has already been applied for radioiodination of antibodies (27) and has been proposed for the radioiodination of small peptides (28). Chemoselective oxime ligation has been successfully applied recently in our laboratory for high-yield $^{18}$F-labeling of a variety of peptides, including the glycosylated octreotide analogs glucose Gluc-S-Dpr($^{[18}\text{F}]$FBOA)TOCA and cellobiose Cel-S-Dpr($^{[18}\text{F}]$FBOA)TOCA (Fig. 1).

The synthesis and radiolabeling of these radioligands, as well as their evaluation in vitro and in vivo are presented in

---


MATERIALS AND METHODS

Peptide Synthesis

General Conditions

Fmoc-(9-fluorenylmethoxycarbonyl-) amino acids as well as N-Boc-aminooxyacetic acid were purchased from Novabiochem (Bad Soden, Germany). Tritylchloride polystyrene resin was obtained from PepChem (Tübingen, Germany). Solvents and all of the other organic reagents and were purchased from Merck Eurolab (Darmstadt, Germany), Alexis (Grünberg, Germany), Aldrich, or Fluka (Neu-Ulm, Germany).

Solid phase peptide synthesis was carried out manually using a flask shaker (St. John Associates Inc., Beltsville, MD).

Analytical reverse-phase (RP)-high-performance liquid chromatography (HPLC) was performed on a Nucleosil 100 C18 (5 µm; 125 × 4.0 mm) column using a Sykam gradient HPLC System (Sykam GmbH, Fürstenfeldbruck, Germany). The peptides were eluted applying different gradients of 0.1% (v/v) trifluoroacetic acid (TFA) in acetonitrile (solvent B) at a constant flow of 1 ml/min (specific gradients are cited in the text). UV detection was performed at 220 nm using a 206 PUD UV-Vis detector (Linear Instruments Corporation, Reno, NV). Both retention times, tR, as well as the capacity factors K’ are cited in the text. K’ is calculated as follows:

\[ K' = \frac{t_R - t_0}{t_0} \]

where

\[ t_R = \text{retention time} \]

and

\[ t_0 = \text{dead volume of the column (ml)} \times \frac{\text{flow (ml/min)}}{10} \]

Preparative RP-HPLC was performed on the same HPLC system using a Multospher 100 RP 18–5 (250 × 10 mm) column at a constant flow of 5 ml/min.

Mass spectra were recorded on the liquid chromatography-mass spectrometry system LCQ from Finnigan (Bremen, Germany) using the Hewlett Packard series 1100 HPLC system.

Synthesis of Small Organic Precursors

S-(2,3,4,6-Tetraacetyl-glucosyl)-Mercaptopropionic Acid Pentafluorophenyl Ester. S-(2,3,4,6-tetraacetyl-glucosyl)-mercaptopropionic acid was synthesized from peracetylated glucose and 3-mercaptopropionic acid using BF3-Et2O as an activator (29). The pentafluorophenyl activated ester was obtained by reaction of the free acid with pentafluorophenol (1.1 eq) in the presence of N,N'-disopropyl carbodiimide (1.1 eq). The overall yield was 62%.

1H NMR (CDCl3): d (ppm) 2.04 (3 H, s), 2.06 (3 H, s), 2.09 (3 H, s), 2.11 (3 H, s), 2.98–3.14 (4 H, m, SCH2CH2), 3.74–3.81 (1 H, m), 4.16–4.30 (2 H, m), 4.60 (1 H, d, J = 10 Hz), 5.05–5.16 (2 H, m), 5.25 (1 H, d, J = 9.5 Hz).

S-1-Heptaacetyl-Celllobiosyl-Mercaptopropionic Acid Pentafluorophenyl Ester. Synthesis was performed in analogy to the glucose-derivative. The overall yield was 48%.

Calculated monoisotopic mass for S-1-heptaacetyl-celllobiosyl-mercaptopropionic acid pentafluorophenyl ester (C35H39O19SF5): 890.3; found: m/z = 913.2 [M+Na]+, 1802.4 [2M+Na]+.

1H-NMR (CDCl3): d (ppm) 1.96 (3 H, s), 1.99 (3 H, s), 2.00 (3 H, s), 2.01 (3 H, s), 2.03 (3 H, s), 2.06 (3 H, s), 2.09 (3 H, s), 2.89–3.05 (4 H, m, SCH2CH2), 3.58–3.67 (2 H, m), 3.71–3.76 (2 H, m), 4.00–4.11 (2 H, m), 4.32–4.39 (1 H, m), 4.48–4.55 (2 H, m), 4.87–5.22 (5 H, m).

Peptide Synthesis

Glycosylated Precursors for Chemoselective Reaction with 4-[18F]Fluorobenzaldehyde

Fmoc-Dpr0-Tyr3-Lys5(Dde)-Ooctreotate. The synthesis of Fmoc-Dpr0-Tyr3-Lys5 (Dde)-octreotate was performed as described previously for TOCA(Dde) (20). Briefly, the peptide sequence Fmoc-Dpr(Boc)-0-Tyr3-Thr(tBu)-Cys(Trt)-Thr(tBu)-D Trp(Boc)-Lys(Dde)-Thr(Boc)-Cys(Trt)-Thr(Boc)-OH was assembled on triethylchloride polystyrene resin according to standard Fmoc protocol. Amino acid coupling was performed using 1-hydroxybenzotriazole (1.5 eq) and O-(1H-benzoazol-1-y1)-N,N,N',N'-tetramethyleneurilium-tetrafluoroborate (1.5 eq) as coupling reagents and (N-ethyl)diisopropylamine (4 eq) as a base. The peptide was cleaved from the solid support using 95% TFA, 2.5% triisobutylicsine, and 2.5% H2O. Disulfide bridge formation was achieved using H2O2 in a tetrahydrofuran/5 mM NH4Ac mixture buffer at pH 7 with saturated NaHCO3. Yield based on resin-bound Thr: 74%.

HPLC (40–100% B in 15 min): tR = 7.8 min; K’ = 3.32. Calculated monoisotopic mass for Fmoc-Dpr0-Tyr3-Lys5 (Dde)-octreotate (C77H92N12O17S2): 1520.6 found: m/z = 1521.7 [M+H]+, 1543.8 [M+Na]+, 1559.6 [M+K]+.

Coupling with N-(Boc)-Aminooxyacetic Acid, Glycosylation, and Deprotection. N-(Boc)-aminooxyacetic acid was coupled to Fmoc-Dpr0-Tyr3-Lys5 (Dde)-octreotate under optimized coupling conditions (1-hydroxy-7-azabenzotriazole, N,N'-disopropyl carbodiimide, and pyridine; Ref. 30). After subsequent Fmoc-deprotection using 20% piperidine in DMF, N2-terminal glycosylation was achieved by reaction with Gluc(4Ac)/S-1-heptaacetyl-celllobiosyl-mercaptopropionic acid pentafluorophenyl ester (1.1 eq) in the presence of N-ethylidisopropylamine (1.1 eq). Dde deprotection of the Lys5 side chain was performed using 2% hydrazine hydrate in DMF, followed by removal of the acetyl protecting groups in the sugar moiety using potassium cyanide in methanol (31). After final Boc-deprotection of the aminooxy-functionality using 50% TFA in dichloromethane (10 min), the peptides were purified using preparative RP-HPLC.

Gluco-S-Dpr(Aoa)-TOCA: HPLC (15–45% B in 15 min): tR = 10.1 min; K’ = 5.17.

Calculated monoisotopic mass for Gluc-S-Dpr(Aoa)-TOCA (C63H87N13O21S3): 1457.5.
DMF, the peptides were isolated using preparative RP-HPLC.

O
ried out using standard conditions (1-hydroxybenzotriazole, Gluc-Lys-TOCA(Dde) and Gluc propionic acid (33) to the unprotected amino functionality of

°
C, the coupling product was purified using RP-HPLC.

nyl-2-[18F]Fluoropropionate

N₉-(1-deoxy-D-fructosyl)-Lys⁶-Tyr⁴-Lys²(Dde)-octreotate [Gluc-Lys-TOCA(Dde)].

Glycosylated Precursors for Reaction with 4-Nitrophenyl-2-[18F]Fluoropropionate

N₉-(1-deoxy-D-fructosyl)-Lys⁶-Tyr⁴-Lys²(Dde)-octreotate [Gluc-Lys-TOCA(Dde)].

Synthesis of the Reference Compounds

FBOA-Derivatives. Gluc-Cel-S-Dpr(Aoa)-TOCA was dissolved in a 1:1 (v/v) mixture of H₂O/methanol, and an equimolar amount of 4-fluorobenzaldehyde was added. After 30 min at 60°C, the coupling product was purified using RP-HPLC.

Gluc-S-Dpr(TBOA)-TOCA: HPLC (20–70% B in 15 min):
tᵣᵣ = 10.2 min; K’ = 5.81.

Calculated monoisotopic mass for Gluc-S-Dpr(TBOA)-TOCA (C₇₀H₆₀N₂₀O₂₈S₃F): 1563.5. found: m/z = 1564.5 [M+H]+, 1602.3 [M+K]+.

Cel-S-Dpr(TBOA)-TOCA: HPLC (20–70% B in 15 min):
tᵣᵣ = 10.0 min; K’ = 5.12.

Calculated monoisotopic mass for Cel-S-Dpr(TBOA)-TOCA (C₇₀H₆₀N₂₀O₂₈S₃F): 1725.6. found: m/z = 1726.6 [M+H]+, 1748.4 [M+Na]+, 1764.3 [M+K]+.

2-Fluoropropionyl Derivatives. Coupling of 2-fluoropropionic acid (33) to the unprotected amino functionality of Gluc-Lys-TOCA(Dde) and Gluc-S-Dpr-TOCA(Dde) was carried out using standard conditions (1-hydroxybenzotriazole, O-(1H-benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium-tetrafluoroborate, and N-ethyl-diisopropylamine). After removal of the Lys⁵-Dde-protecting group using 2% hydrazine hydrate in DMF, the peptides were isolated using preparative RP-HPLC.

Gluc-Lys(N₉-2-fluoropropionyl)-TOCA.


Gluc-S-Dpr(N₉-2-fluoropropionyl)-TOCA (Gluc-S-Dpr-(FP)TOCA).


Radiolabeling

Instrumentation

Radio-HPLC was performed using a Sykam gradient system (Sykam GmbH, Fürstenfeldbruck, Germany) and an UVIS 200 photometer (Linear Instruments Corporation, Reno, NV). For radioactivity measurement, the outlet of the UV-photometer was connected to a Na(Tl) well-type scintillation counter Ace Mate 925-Scint (EG & G Ortec, München, Germany).

18F-Labeling Using 4-[18F]Fluorobenzaldehyde

The synthesis of 4-[18F]fluorobenzaldehyde from 4-formyl-N,N,N-trimethylammonium triflate was performed as described previously (34). For peptide labeling, a solution of Gluc/Cel-S-Dpr(Aoa)-TOCA (0.11 μmol) in 100 μl water was added to 500 μl of a freshly prepared methanolic 4-[18F]fluorobenzaldehyde solution. Trifluoracetic acid (2%; 8 μl) was added to adjust the pH to 2. After 10 min at 60°C and preparative HPLC [column: YMC RP 18 s-4 μm 80A, 150 × 20 mm (YMC Europe), gradient: 0% B for 5 min, then 15–50% B in 20 min, 10 ml flow] overall radiochemical yields (not optimized) of Gluc/Cel-S-Dpr([18F]FBOA)-TOCA were about 40–60% in an overall synthesis time of ~50 min.

18F-Labeling Using 4-Nitrophenyl 2-[18F]Fluoropropionate

The synthesis of 4-nitrophenyl 2-[18F]fluoropropionate was carried out according to the literature (14). For 18F-acetylation, the respective Lys⁵(Dde)-protected peptide (1.3–1.5 mg) was dissolved in 180–200 μl of DMSO, and 2 μl of N-ethyl-diisopropylamine were added. The resulting solution was added to a vial containing no carrier added (n.c.a) 4-nitrophenyl 2-[18F]fluoropropionate. The mixture was heated to 70°C for 15 min. After analytical HPLC reaction control, 4 μl of hydrazine hydrate were added to the solution containing the [18F]fluoropropionate. The deprotected peptide was then isolated via preparative HPLC (Multosher 100 RP18–5, 250 × 10 mm; linear gradient 22–30% B in 30 min, flow rate 5 ml/min, tᵣᵣ = 15–17 min; K’ = 7).

Radiochemical yields (based on 4-nitrophenyl 2-[18F]fluoropropionate) of N₉-(1-deoxy-D-fructosyl)-N₉-(2-[18F]fluoropropionyl)-Lys⁵-Tyr⁴-octreotate [Gluc-Lys([18F]FP)-TOCA] and Gluc-S-Dpr(N₉-2-[18F]fluoropropionyl)-TOCA Gluc-S-Dpr([18F]FP)TOCA were ~25% in a synthesis time of 1 h.
Radioiodination

Radioiodination of Gluc-S-Dpr(FP)TOCA, Gluc-S-Dpr-(FBOA) BOA)TOCA, Cel-S-Dpr(FBOA)-TOCA, and the reference TOC was performed using the iodogen method.

A solution of 100–200 µg of peptide in 200 µl of PBS (0.1 M; pH 7.4) was transferred to an Eppendorf cap coated with 30 µg of iodogen (Pierce, Rockford, IL). After the addition of 5–20 µl of solution of radiodiode (Amersham, Buckinghamshire, United Kingdom) in 0.05 M NaOH [(125)I]NaI (n.c.a.); 18–74 MBq, [(125)INaI (H11003)]: 37–185 MBq] the cap was vortexed, and the labeling reaction was allowed to proceed for 15–20 min at room temperature. The peptide solution was then removed from the insoluble oxidizing agent.

The radioiodinated peptides were purified via RP-HPLC [column: Nucleosil 100 C18, 5 µm, 125 × 4.0 mm (CS GmbH, Langerwehe, Germany); flow rate: 1 ml/min]. Isocratic solvent mixtures containing 28% [(125)ITOC], 32% [(125)I-Gluc-S-Dpr(FP)TOCA], 41% [(125)I-Cel-S-Dpr(FBOA)TOCA], or 42% [(125)I-Gluc-S-Dpr(FBOA)TOCA] ethanol (0.5% AcOH) in water (0.5% AcOH) were used. Radiochemical yields after purification ranged from 45% to 65%. Radiochemical purities were >98% for all of the radioiodinated agents.

For the paired label internalization experiments, the collected HPLC fractions of both [(125)ITOC] and the respective [(125)ITOC]-labeled radioligand were each evaporated to dryness. The radiopeptides were redissolved in assay medium (containing 5–20% FCS (Seromed) and 2 mM L-glutamine (Gibco BRL, Life Technologies, Inc., Karlsruhe, Germany). Cultures were maintained at 37°C in a 5% CO2/humidified air atmosphere.

In the assay medium used for the internalization studies, FCS was replaced by 1% BSA (Sigma, St. Louis, MO).

For cell counting, a CASY 1-TT cell counter and analyzer system (Schärfe System GmbH, Reutlingen, Germany) was used.

**Internalization and Determination of EC50,R**

Experiments were performed as described in detail previously. Briefly, after preconditioning of the cells (~100,000 cells/well) with 190 µl of unsupplemented medium for a minimum of 15 min, 50 µl (per well) of DMEM (5% BSA) containing increasing concentrations of unlabeled TOC were added, followed by the addition of ~100,000 cpm of both the respective [(125)I]-labeled glycosylated peptide and of the reference [(125)ITOC] in 10 µl of DMEM (5% BSA). Final TOC-concentrations in the incubation medium used for the determination of the EC50,R were 0.1, 0.2, 0.5, 0.8, 1, 2, 4, 6, 8, 10, 12, 15, 20, 50, 100, 500, and 1000 nM. In a control experiment (n.c.a. conditions), TOC-free DMEM (5% BSA) was added. Nonspecific internalization was determined by including 5 µM unlabeled TOC. Experiments were carried out in triplicate for each concentration. Because internalization of [(125)ITOC] and its analogs into the CHO cells used is very fast (up to 15–35% of the applied activity within 10 min), the incubation time used for all of the internalization experiments described here has also been limited to 10 min.

After incubation at 37°C, the incubation medium was removed, and cells were rinsed with 250 µl of fresh medium. The combined medium fractions represent the amount of free radioligand. Receptor-bound (acid releasable) radioactivity was then removed using 2 × 250 µl of ice cold acid wash buffer (0.02 M NaOAc buffered with AcOH to pH = 5). The internalized activity was released by incubation with 250 µl of 1 N NaOH, transferred to vials, and combined with 250 µl of PBS used for rinsing the wells. Quantification of the amount of free, acid-releasable, and internalized activity was performed in a gamma counter.

For numerical analysis of the EC50 of TOC for internalization, data for both the [(125)I]-labeled compound of interest and for the reference [(125)ITOC] in the same experiment were first corrected by the amount of nonspecific internalization, respectively, and then each normalized to the amount of internalized ligand in the absence of unlabeled competitor (100%). Data were fitted with a weighted two-parameter logistic function using SigmaPlot. To eliminate the influence of cell count and cell viability on the absolute EC50-values, data are expressed as the ratio (EC50,R) of the EC50 observed for the compound of interest and the EC50 found for [(125)ITOC] in the same experiment [EC50,R = EC50 (compound of interest)/EC50 ([(125)ITOC])].

Externalization and Recycling

As in the previous experiment, cells were incubated with the respective \(^{125}\)I-labeled peptide and the reference \(^{125}\)I-TOC at 37°C for 10 min and washed with medium. To ensure receptor integrity, no acid wash was performed after this initial internalization incubation. Then, to determine the extent of ligand recycling, two different experiments were performed. In the experiment allowing ligand recycling, 200 µl of assay medium and 50 µl of medium (5% BSA) were added to each well. In the experiment inhibiting ligand recycling, 200 µl of assay medium and 50 µl of medium (5% BSA) containing 25 µM TOC were added to each well. Experiments were carried out in triplicate for both experimental conditions. Subsequently, the cells were incubated at 37°C for 30 and 60 min, respectively. The supernatant was removed and combined with 250 µl of medium used for rinsing the cells. This fraction represents the amount of externalized ligand. The following acid wash and lysis of the cells was performed as described for the internalization experiment.

Affinity Profiles to lst

Cell membrane pellets of cells stably expressing human sst\(_1\), sst\(_2\), sst\(_3\), sst\(_4\), and sst\(_5\) (sst\(_1\) and sst\(_5\): CHO-K1 cells; sst\(_2\)–sst\(_4\): CCL39 cells) were prepared, and receptor autoradiography was performed on pellet sections (mounted on microscope slides), as described in detail previously (36). Displacement experiments were performed with the universal somatostatin radioligand \(^{125}\)I-[Leu\(^8\), D-Trp\(^22\), Tyr\(^25\)]-somatostatin 28 using increasing concentrations of Gluc-Lys\(^{[18}F\)FP\]TOCA, Gluc-S-Dpr\(^{[18}F\)FP\]TOCA, Gluc-S-Dpr\(^{[18}F\)FBOA\]TOCA, Cel-S-Dpr\(^{[18}F\)FBOA\]TOCA, 3-iodo-Tyr\(^3\)-octreotide, and the reference somatostatin 28 (0.1 nm) were performed with the universal somatostatin radioligand.

In Vivo Studies

Tumor Model

The AR42J cell line as a transplantable rat pancreatic tumor model with high sst\(_5\) expression (37) was used. To establish tumor growth, cells were detached from the surface of the culture flasks using 1 mM EDTA in PBS, centrifuged, and resuspended in serum-free culture medium. Concentration of the cell suspension was 2.5–5 × 10\(^6\) cells/100 µl serum. Nude mice (female, 6–8 weeks) were injected with 100 µl of the cell suspension s.c. into the flank. Ten days after tumor transplantation all of the mice showed solid palpable tumor masses (tumor weight 200–900 mg) and were used for the experiments.

Biodistribution Studies

The radiolabeled peptides [40–60 µCi in 100 µl PBS (pH 7.4)], were injected i.v. into the tail vein of AR42J tumor-bearing nude mice. For competition studies, 0.4 or 0.6 mg/kg TOC (15 µg/mouse) were coinjected. The animals (groups of 3–5) were sacrificed 10 and 60 min after injection, and the organs of interest were dissected. The radioactivity was measured in weighted tissue samples using a gamma counter.

PET Imaging

For PET imaging, the \(^{18}\)F-labeled peptides [35–77 µCi in 100–200 µl PBS (pH 7.4)], were injected i.v. into the tail vein of AR42J tumor-bearing nude mice. For competition studies, 0.6 mg/kg TOC (15 µg/mouse) were coinjected. After 60 min, mice were either euthanized (Gluc-S-Dpr\(^{[18}F\)FP\]TOCA) or anesthetized using a xylazine/ketamine combination. Static PET images (20 min; 5 min transmission; zoom 3.0) were acquired using an Siemens Exact HR Plus scanner. The axial resolution of the scanner at full-width at half-maximum is ~5 mm, the transaxial resolution is ~8 mm. Images were reconstructed by iterative reconstruction (8 iterations, 4 subsets). For the quantification of tumor:kidney ratios, ROI [region of interest] analysis was performed in a minimum of 2 coronal slices/mouse.

RESULTS

Synthesis. Standard Fmoc solid phase peptide synthesis afforded TOCA(Dde) in yields >85% (based on resin-bound Thr\(^1\)). After quantitative N-terminal solution phase coupling with Fmoc-Lys(Boc) or Fmoc-Dpr(Boc) and subsequent Fmoc-deprotection, the peptides were either glycosylated via Amadori reaction (Gluc-Lys-TOCA(Dde)) or via conjugation with the pentafluorophenyl active esters of S-(tetraacetylglycosyl)- or S-(heptaacetyl-cellbiosyl)-3-mercaptopropionic acid. As determined via RP-HPLC reaction control, yields of Amadori-glycosylation never exceeded 80% within 16 h at 60°C, whereas the latter reaction was quantitative within 30–45 min at room temperature.

Initially, Dpr-sidechain acylation with Boc-protected 2-aminoxyacetic acid \([N-(Boc)-aminoxyacetic acid]\) was performed using a large excess (10 eq) of \(N\)-(Boc)-aminoxyacetic acid and coupling reagents [1-hydroxybenzotriazole/\(N,N,O,O\)-tetramethyluronium-tetrafluoro- borate] over Dpr-deprotected peptide. Even under these conditions, coupling yields were comparably low (<80% as determined via HPLC reaction control). In contrast, using 1-hydroxy-7-azabenzotriazole/\(N,N\)-disopropyl carbodiimide (1.5 eq) as coupling reagents and pyridine as a base (30) led to quantitative \(N\)-(Boc)-aminoxyacetic acid-functionalization.

Whereas both precursors for \(^{18}\)Ffluoropropionylation, Gluc-Lys-TOCA(Dde) and Gluc-S-Dpr-TOCA(Dde), were obtained in yields of ~20% (based on nonglycosylated peptide) after deprotection and purification via preparative HPLC, yields of Aoa-functionalized peptides were low (2–5%). Due to the high reactivity of the Aoa-group (38), side product formation during preparative HPLC necessitated additional purification steps and reduced final product purity (UV trace in HPLC at 220 nm) to 80–85%.

Radiolabeling. Both n.c.a. 4-nitrophenyl-2-[\(^{18}\)F]fluoropropionate (14) and n.c.a. 4-[\(^{18}\)F]fluorobenzaldehyde (34; see footnote 5) were synthesized as described. After \(^{18}\)Ffluorocyclization, in situ Dde-deprotection, and subsequent RP-HPLC purification, radiochemical yields of Gluc-Lys\(^{[18}F\)FP\]TOCA and Gluc-S-Dpr\(^{[18}F\)FP\]TOCA based on 4-nitrophenyl-2-[\(^{18}\)F]fluoropropionate ranged from 25% to 45% (d.c.), whereas one-step conjugation with 4-[\(^{18}\)F]fluorobenzaldehyde, followed by RP-HPLC isolation, afforded Gluc-S-Dpr\(^{[18}F\)FBOA\]TOCA and Cel-S-Dpr\(^{[18}F\)FBOA\]TOCA.
FBOA)TOCA in 60–80% radiochemical yield. Overall preparation times for the [18F]FP- and the [18F]FBOA-labeled peptides were 3 h and 50 min, respectively. Radiochemical purities of all of the 18F-labeled peptides (n.c.a.) were >98%.

Usually, the amount of 18F-labeled peptide produced was below the UV-detection limit during HPLC, which impeded estimation of the specific activity of the respective radioligand. Because this is indispensable for an approximate estimation of the concentration of radioligand used in the internalization assays, however, not the 18F-labeled compounds, but the corresponding 123I-labeled 19F-reference compounds Gluc-S-Dpr(FP)TOCA, Gluc-S-Dpr(FBOA)TOCA, and Cel-S-Dpr(FBOA)TOCA were used in the in vitro experiments. This strategy not only affords radioligands with comparable specific activity but, furthermore, ensures comparability of the data obtained for the new fluoronanologs with those obtained for other radiiodinated glyco-analogs of octreotide in a previous study.6 On the basis of the fairly similar sst2-affinities of TOC and radiiodinated TOC (Table 2), an effect of radioiodination on the internalization characteristics of the 123I-labeled fluoronanologs investigated was assumed to be negligible.

Radioiodination of Gluc-S-Dpr(FP)TOCA, Gluc-S-Dpr(FBOA)TOCA, and Cel-S-Dpr(FBOA)TOCA, as well as the reference TOC for the in vitro internalization assays afforded the respective 123I-labeled peptides in radiochemical yields of 45–75% after RP-HPLC purification. All of the radioiodinated peptides were obtained in high radiochemical purity (>99%). Because the HPLC conditions applied allowed efficient separation of the radioiodinated product from the unlabelled precursor (ΔtR ≥ 4 min) for all of the radioiodinated analogs investigated, and because no coeluting carrier peak was observed in the quality control UV-chromatogram, the specific activity of the labeled peptide was assumed to be that of the radioiodide used for their preparation (≈2000 Ci/mmol for 125I, ≈5000 Ci/mmol for 123I).

**Lipophilicity.** Lipophilicities of the four compounds investigated are listed in Table 1. Despite the loss of the positive charge on Nα of o-Phe1 (Fig. 1), the lipophilicity of Gluc-S-Dpr([18F]FP)TOCA is substantially lower than that of Gluc-Lys([18F]FP)TOCA. Substitution of the small [18F]FP moiety by the aromatic [18F]FBOA residue, however, leads to a strongly increased lipophilicity of Gluc-S-Dpr([18F]FBOA)TOCA compared with Gluc-S-Dpr([18F]FP)TOCA (log Pcow = −1.24 ± 0.03 versus −2.80 ± 0.01, respectively). This effect is partly compensated by replacement of the monosaccharide glucose by the more hydrophilic disaccharide cellobiose in Cel-S-Dpr([18F]FBOA)TOCA, leading to an intermediate log Pcow identical to the value found for Gluc-Lys([18F]FP)TOCA.

**In Vitro Studies.** For all four of the 18F-labeled peptides investigated the in vitro binding profile to human sst1,2 has been determined (Table 2). The corresponding 18F reference compounds all showed very high hsst1 affinity comparable with that of native somatostatin 28, including no affinity to hsst2, very low affinity to hsst3, and moderate affinity to hsst4 and hsst5. Compared with iodinated Tyr3-octreotide, which is used as the internal reference in the dual tracer internalization studies performed in this study, the hsst1 affinity of Gluc-Lys(FP)-TOCA, Gluc-S-Dpr(FP)TOCA, and Gluc-S-Dpr(FBOA)TOCA is decreased by a factor of two, whereas it is comparable for the Cel-S-Dpr(FBOA) analog. Of the four compounds, Gluc-Lys(FP)-TOCA showed the highest and Cel-S-Dpr(FBOA)-TOCA the lowest sst2-receptor specificity.

For [123I]Gluc-S-Dpr(FP)TOCA, [123I]Gluc-S-Dpr(FBOA)TOCA, and [123I]Cel-S-Dpr(FBOA)TOCA the amount of activity internalized into hsst2-expressing CHO cells was determined in dual tracer experiments (internal reference = [125I]TOC) both in the absence (control) or the presence of increasing concentrations of unlabelled TOC (0.1–1000 nM; Fig. 2). Data were corrected by the nonspecific internalization (determined by coinubcation with 5 μM TOC) and normalized to maximum internalization (control = 100%). The higher the amount of cold TOC needed to reduce cellular uptake of the respective 123I-labeled compound as well as the internal reference [123I]TOC to 50% of maximum internalization (EC50 for internalization), the higher, in a first approximation, the receptor affinity of the ligand. To eliminate interexperimental inaccuracies in cell count or cell viability, data are generally cited

---

**Table 1** Lipophilicities (log Pcow) of the four 18F-labeled TOCA analogs investigated in this study

<table>
<thead>
<tr>
<th>Peptide</th>
<th>log Pcow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluc-Lys([18F]FP)TOCA</td>
<td>−1.70 ± 0.03</td>
</tr>
<tr>
<td>Gluc-S-Dpr([18F]FP)TOCA</td>
<td>−2.80 ± 0.01</td>
</tr>
<tr>
<td>Gluc-S-Dpr([18F]FBOA)TOCA</td>
<td>−1.24 ± 0.03</td>
</tr>
<tr>
<td>Cel-S-Dpr([18F]FBOA)TOCA</td>
<td>−1.70 ± 0.03</td>
</tr>
</tbody>
</table>

**Table 2** Affinity profiles (IC50) to the human sst1-sst5 receptors determined for somatostatin 28 and the compounds investigated using 125I-[Leu6, D-Trp22, and Tyr3]somatostatin 28 as the radioligand

<table>
<thead>
<tr>
<th>Peptide</th>
<th>sst1</th>
<th>sst2</th>
<th>sst3</th>
<th>sst4</th>
<th>sst5</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS-28</td>
<td>3.6 ± 0.3 (5)</td>
<td>2.1 ± 0.3 (5)</td>
<td>3.2 ± 0.2 (5)</td>
<td>3.0 ± 0.3 (5)</td>
<td>2.5 ± 0.2 (5)</td>
</tr>
<tr>
<td>TOC</td>
<td>&gt;1,000 (2)</td>
<td>3.1 ± 1.8 (2)</td>
<td>330 ± 146 (2)</td>
<td>346 ± 114 (2)</td>
<td>10.5 ± 1.8 (2)</td>
</tr>
<tr>
<td>1-TOC</td>
<td>&gt;10,000 (3)</td>
<td>1.3 ± 0.3 (3)</td>
<td>128 ± 22 (3)</td>
<td>867 ± 33 (3)</td>
<td>50 ± 12 (3)</td>
</tr>
<tr>
<td>Gluc-Lys([18F]FP)TOCA</td>
<td>&gt;10,000 (3)</td>
<td>2.8 ± 0.4 (3)</td>
<td>&gt;1,000 (3)</td>
<td>437 ± 84 (3)</td>
<td>123 ± 8.8 (3)</td>
</tr>
<tr>
<td>Gluc-S-Dpr([18F]FP)TOCA</td>
<td>&gt;1,000 (2)</td>
<td>2.7 ± 2.1 (2)</td>
<td>742 ± 182 (2)</td>
<td>339 ± 11 (2)</td>
<td>87 ± 18 (2)</td>
</tr>
<tr>
<td>Gluc-S-Dpr([18F]FBOA)TOCA</td>
<td>&gt;1,000 (2)</td>
<td>2.5 ± 1.3 (2)</td>
<td>692 ± 108 (2)</td>
<td>298 ± 52 (2)</td>
<td>74 ± 20 (2)</td>
</tr>
<tr>
<td>Cel-S-Dpr([18F]FBOA)TOCA</td>
<td>&gt;1,000 (2)</td>
<td>1.8 ± 1.2 (2)</td>
<td>707 ± 30 (2)</td>
<td>339 ± 163 (2)</td>
<td>45 ± 1 (2)</td>
</tr>
</tbody>
</table>

**Note:** SS 28, somatostatin 28.
as the normalized EC$_{50,R}$ = EC$_{50}$($[^{125}]$I-Cel-S-Dpr(FBOA)-TOCA)/EC$_{50}$($[^{125}]$I-TOC; n = 3).

As summarized in Table 3, both $[^{123}]$Gluc-S-Dpr(FBOA)-TOCA and $[^{125}]$Cel-S-Dpr(FBOA)-TOCA show substantially enhanced internalization compared with $[^{125}]$I-TOC (140–160%). For both analogs, increased internalization is paralleled by very high EC$_{50,R}$ values (3.2–4.1). In contrast, $[^{123}]$I-Gluc-S-Dpr(FP)-TOCA shows the highest internalization of the compounds investigated (190.2 ± 6.6% of the reference) but a comparably low EC$_{50,R}$.

To assess the extent of ligand externalization and subsequent reinternalization, i.e., ligand recycling, cells were first incubated with the respective radioligands for 10 min to allow intracellular ligand accumulation. After replacement of the incubation medium with ligand-free medium, the amount of activity released into the medium within 60 min was nearly identical (21–29% of the activity that was cellularly accumulated 60 min p.i. was located after the internalization incubation; data not shown). When recycling was inhibited, 96–97% of the cellular activity were externalized into the medium in the case of all of the $^{125}$I-labeled sugar analogs ($[^{125}]$I-TOC: 92–93%) over the 60-min observation period.

**In Vivo Studies.** The biodistribution of the four $^{18}$F-labeled TOCA analogs 10 and 60 min postinjection (p.i.) was determined in AR42J tumor-bearing nude mice (Table 4).

All of the compounds showed rapid elimination from the blood pool and were mainly cleared via the kidneys. Activity levels in the kidneys 60 min p.i. were comparable for Gluc-Lys($[^{18}]$FP)-TOCA and both $^{18}$F-FBOA analogs (7.49–9.82% of injected dose/g; %ID/g). In contrast, Gluc-S-Dpr($[^{18}]$FP)-TOCA showed a particularly low renal activity accumulation of 1.86 ± 0.70%ID/g.

This derivative also exhibited the lowest nonspecific uptake in liver and intestine (0.23 ± 0.04 and 1.11 ± 0.13%ID/g at 60 min p.i., respectively). Whereas hepatic and intestinal accumulation 60 min p.i. were slightly higher and nearly identical for Gluc-Lys($[^{18}]$FP)-TOCA and Cel-S-Dpr($[^{18}]$FBOA)-TOCA, it was significantly increased in the case of Gluc-S-Dpr($[^{18}]$FP)-TOCA (3.54 versus 0.72–0.88 and 6.96 versus 1.54–1.56%ID/g, respectively).

In sst-positive tissues such as stomach, pancreas, and adrenals, a decrease in activity concentration was observed between 10 and 60 min p.i. for all of the peptides, indicating a certain contribution of circulating activity to the observed uptake in

**Fig. 3** Exemplary externalization of $[^{123}]$I-Cel-S-Dpr(FBOA)-TOCA and the reference $[^{123}]$I-TOC from Chinese hamster ovary cells stably expressing hss$_{2}$ under conditions allowing (no TOC in the external medium) and inhibiting (5 μM TOC in the external medium) ligand recycling (n = 3); bars, ±SD.

**Table 3** Internalization (in % of the reference $[^{125}]$I-TOC) of the compounds investigated in CHO cells stably expressing hss$_{2}$ and the EC$_{50,R}$ = EC$_{50}$($[^{125}]$I-labeled analog)/EC$_{50}$($[^{125}]$I-TOC) of unlabeled TOC for internalization.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Internalization [% of $[^{125}]$I-TOC]</th>
<th>EC$_{50,R}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{123}]$Gluc-S-Dpr(FP)TOCA</td>
<td>190.2 ± 6.6 (3)</td>
<td>3.21 ± 0.12 (3)</td>
</tr>
<tr>
<td>$[^{123}]$Gluc-S-Dpr(FBOA)TOCA</td>
<td>139.3 ± 2.4 (2)</td>
<td>3.24 ± 0.13 (2)</td>
</tr>
<tr>
<td>$[^{123}]$Cel-S-Dpr(FBOA)TOCA</td>
<td>162.6 ± 7.8 (3)</td>
<td>4.10 ± 0.17 (3)</td>
</tr>
</tbody>
</table>

*CHO, Chinese hamster ovary.*
Dpr(\([^{18}\text{F}]\text{FBOA}\) TOCA and in particular BOA-pair at both time points. However, the tumor uptake Dpr(\([^{18}\text{F}]\text{FBOA}\) TOCA with its not only observed for Cel high-contrast PET imaging of sst-expressing malignancies, were simulation of all four of the analogs increased with time. It was respectively).

In contrast to the other sst-expressing tissues, tumor accumulation of all four of the analogs increased with time. It was comparable both for the \([^{18}\text{F}]\text{FP}\)-pair as well as for the \([^{18}\text{F}]\text{FBOA}\)-pair at both time points. However, the tumor uptake observed for Gluc-Lys(\([^{18}\text{F}]\text{FP}\) TOCA and in particular for Cel-S-Dpr(\([^{18}\text{F}]\text{FBOA}\) TOCA was significantly higher than that of the two \([^{18}\text{F}]\text{FP}\)-analogs (21.8 and 24.0 \%ID/g at 60 min p.i., respectively).

High tumor:nontumor ratios, which are indispensable for high-contrast PET imaging of sst-expressing malignancies, were not only observed for Cel-S-Dpr(\([^{18}\text{F}]\text{FBOA}\) TOCA with its very high tumor accumulation (24.0 \pm 2.5 \%ID/g at 60 min p.i.) but were even higher for Gluc-S-Dpr(\([^{18}\text{F}]\text{FP}\) TOCA (15.1 \pm 1.5 \%ID/g at 60 min p.i.; Fig. 4). Due to the particularly low activity levels found in blood, liver, intestine, and kidney for Gluc-S-Dpr(\([^{18}\text{F}]\text{FP}\) TOCA, tumor:organ ratios 60 min p.i. were 123:1, 66:1, 14:1, and 8:1, respectively, for these organs. For Cel-S-Dpr(\([^{18}\text{F}]\text{FBOA}\) TOCA, values were 42:1, 27:1, 15:1, and 3:1 for blood, liver, intestine, and kidney, respectively.

The tumor: kidney ratios determined via ROI analysis of PET images obtained 60 min p.i. of Gluc-S-Dpr(\([^{18}\text{F}]\text{FP}\) TOCA, Gluc-S-Dpr(\([^{18}\text{F}]\text{FBOA}\) TOCA, and Cel-S-Dpr(\([^{18}\text{F}]\text{FBOA}\) TOCA (Fig. 5) are in accordance with the data determined in the biodistribution studies (7.2 versus 8.1, 1.9 versus 2.2, and 1.4 versus 3.2, respectively).

To determine the specificity of radioligand accumulation in tumor and other sst-expressing tissues, the biodistribution of all four of the \([^{18}\text{F}]\text{-labeled TOCA analogs was also determined 60 min after injection of 15 \mu\text{g of unlabelled TOC. Under these conditions, tumor accumulation of the four derivatives investigated was reduced to 18\% (Gluc-S-Dpr(\([^{18}\text{F}]\text{FP}\) TOCA and Cel-S-Dpr(\([^{18}\text{F}]\text{FBOA}\) TOCA), 26\% (Gluc-S-Dpr(\([^{18}\text{F}]\text{FBOA}\) TOCA), and 30\% (Gluc-Lys(\([^{18}\text{F}]\text{FP}\) TOCA of control (Fig. 6), demonstrating a high extent of somatostatin receptor-mediated uptake. In

Table 4 Biodistribution of Gluc-Lys(\([^{18}\text{F}]\text{FP}\) TOCA, Gluc-S-Dpr(\([^{18}\text{F}]\text{FP}\) TOCA, Gluc-S-Dpr(\([^{18}\text{F}]\text{FBOA}\) TOCA, and Cel-S-Dpr(\([^{18}\text{F}]\text{FBOA}\) TOCA in AR42J rat pancreatic tumor bearing nude mice 10 and 60 min p.i. (n = 4–5)

<table>
<thead>
<tr>
<th>Gluc-Lys(([^{18}\text{F}]\text{FP}) TOCA</th>
<th>Gluc-S-Dpr(([^{18}\text{F}]\text{FP}) TOCA</th>
<th>Gluc-S-Dpr(([^{18}\text{F}]\text{FBOA}) TOCA</th>
<th>Cel-S-Dpr(([^{18}\text{F}]\text{FBOA}) TOCA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 min p.i.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>0.54 \pm 0.06</td>
<td>3.15 \pm 1.09</td>
<td>5.58 \pm 0.53</td>
</tr>
<tr>
<td>Liver</td>
<td>0.72 \pm 0.14</td>
<td>1.57 \pm 0.58</td>
<td>3.54 \pm 0.88</td>
</tr>
<tr>
<td>Intestine</td>
<td>1.88 \pm 0.52</td>
<td>1.11 \pm 0.13</td>
<td>2.69 \pm 0.49</td>
</tr>
<tr>
<td>Kidney</td>
<td>8.69 \pm 1.09</td>
<td>1.86 \pm 0.70</td>
<td>9.82 \pm 2.97</td>
</tr>
<tr>
<td>Spleen</td>
<td>n.d.</td>
<td>0.11 \pm 0.03</td>
<td>0.56 \pm 0.14</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.24 \pm 0.03</td>
<td>0.05 \pm 0.03</td>
<td>0.46 \pm 0.43</td>
</tr>
<tr>
<td>Lung</td>
<td>n.d.</td>
<td>0.51 \pm 0.08</td>
<td>1.97 \pm 0.39</td>
</tr>
<tr>
<td>Stomach</td>
<td>3.07 \pm 0.14</td>
<td>1.44 \pm 0.14</td>
<td>0.52 \pm 0.09</td>
</tr>
<tr>
<td>Pancreas</td>
<td>2.32 \pm 0.11</td>
<td>0.11 \pm 0.03</td>
<td>0.00 \pm 0.00</td>
</tr>
<tr>
<td>Adrenals</td>
<td>0.86 \pm 0.20</td>
<td>0.18 \pm 0.27</td>
<td>0.23 \pm 0.05</td>
</tr>
<tr>
<td>Tumor</td>
<td>13.54 \pm 1.47</td>
<td>9.63 \pm 2.18</td>
<td>15.08 \pm 1.45</td>
</tr>
</tbody>
</table>

*p.i., postinjection; n.d., not determined.

Fig. 4 Tumor:organ ratios of [Gluc-Lys(\([^{18}\text{F}]\text{FP}\) TOCA, Gluc-S-Dpr(\([^{18}\text{F}]\text{FP}\) TOCA, Gluc-S-Dpr(\([^{18}\text{F}]\text{FBOA}\) TOCA, and Cel-S-Dpr(\([^{18}\text{F}]\text{FBOA}\) TOCA in AR42J tumor-bearing nude mice 60 min p.i. (n = 4–5); bars, \pm SD.
pancreas and stomach, activity levels were reduced to a lower extent (38–49% and 30–61% of control, respectively), whereas in adrenals and lung, organs known to also express somatostatin receptors, ligand accumulation was even increased under blocking conditions (up to 240% of control; data not shown). The effect of coadministration of a blocking dose of TOC was also demonstrated in PET studies using a conventional PET scanner (Fig. 5).

**DISCUSSION**

On the basis of its pharmacokinetic profile, Gluc-Lys([18 F]FP)-TOCA is ideally suited for in vivo somatostatin receptor imaging using PET. It shows rapid renal elimination from the circulation and very high tumor accumulation even at early time points after injection (<30 min; Ref. 22). However, clinical routine applicability of PET radiopharmaceuticals does not only rely on their in vivo imaging properties but also on the possibility of fast and high-yield radiosynthesis, prerequisites that are not fulfilled for Gluc-Lys([18 F]FP)-TOCA. Radiochemical yields of the five-step synthesis are comparably low (20–30%), partly due to long reaction times (~3 h). The recent development of a high-yield two-step radiohalogenation method via chemoselective oxime formation between an aminooxy-functionalized peptide and a radiohalogenated aldehyde or ketone (28; see footnote 5) offers new perspectives for large-scale radiofluorination of octreotide analogs and other bioactive peptides for clinical PET studies. Therefore, it was the aim of this study to exploit the advantages of this new [18 F]-labeling methodology as well as the potency of glycosylation as a chemical tool to improve peptide pharmacokinetics (16–22; see footnote 4) for the development of new carbohydrated [18 F]-labeled octreotide derivatives suitable for routine application in PET imaging of sst-overexpressing human malignancies.

The evaluation of the three new [18 F]-labeled TOCA derivatives presented in this study, Gluc-S-Dpr([18 F]FP)-TOCA, Gluc-S-Dpr([18 F]FBOA)-TOCA, and Cel-S-Dpr([18 F]FBOA)-TOCA (Fig. 1), in comparison with the reference Gluc-Lys([18 F]FP)-TOCA, was focused on several aspects, including ease of precursor synthesis and radiolabeling, and influence of both carbohydrate- and radiolabeling method on in vitro ligand internalization and residualization in sst-expressing cells and in vivo pharmacokinetics.

From a synthetic and structural point of view, glycosylation via Maillard reaction and subsequent Amadori rearrangement used in the case of Gluc-Lys([18 F]FP)-TOCA has the inherent disadvantage of affording a variety of glycosylated conformers. In Gluc-Lys([18 F]FP)-TOCA, the α- and β-furanoid, as well as the open-chain conformations of the deoxy-ketoses are possible beside the β-pyranoid structure depicted in Fig. 1 and may be present in equilibrium mixtures (39). It has been demonstrated,
however, that in the case of radioiodinated TOC and TOCA derivatives glycosylation via Amadori reaction not only allows “fine tuning” of the lipophilicity of the ligands and, thus, excretion characteristics. In contrast to their maltose and maltotriose counterparts, the glucose Amadori products ([123I]Gluc-TOC and [123I]Gluc-TOCA) exhibited significantly enhanced sst2-mediated ligand internalization in vitro. In vivo, both [123I]Gluc-TOCA and especially the maltotriose analog [123I]Mtr-TOCA showed substantially increased tumor accumulation compared with [123I]TOCA (22).

For a more precise assessment of the influence of carbohydrate structure on the physicochemical and pharmacokinetic properties of radiolabeled octreotide analogs, a glycosylation method yielding glycopeptides with known sugar conformation was needed. This prerequisite was met by the use of S-glycosylated 3-mercaptopropionic acid as a glycosyl donor (40). Use of sugar 1,2-trans peracetate precursors results in the formation of the β-thioglycoside exclusively and, thus, glycopeptides with known sugar conformation. Furthermore, compared with glycosylation via Amadori reaction, peptide conjugation with S-glycosylated 3-mercapto propionic acid has the distinct advantage of a much greater ease of synthesis, i.e., shorter reaction times (30 min versus 16 h), higher glycosylation yields (>99% versus ~80%), and a significantly lower proportion of side product formation.

With respect to the efficiency of peptide 18F-labeling, the present as well as previous studies (see footnote 5) clearly illustrate the excellence of chemoselective oxime formation over other prosthetic group radiofluorination strategies (13). The present method allows large-scale radiofluorination of peptides in high yields and offers unprecedented flexibility both with respect to the radiohalogenation precursor as well as to the nature of the radiohalogenated aldehyde or ketone used.

On a clinical basis, the applicability of 18F-labeled octreotide analogs for high contrast somatostatin receptor imaging using PET crucially relies on three factors, including fast and efficient radioligand accumulation in target tissue, sufficient ligand retention, and rapid elimination from the circulation and nontarget organs. Whereas the latter is greatly influenced by general physicochemical properties of the radioligand molecule such as lipophilicity or net charge, the first two mainly depend on the efficiency of receptor-mediated ligand internalization.

The amount of internalized receptor-agonist complex can be influenced both on the level of cellular receptor regulation and on the basis of radioligand structure. It has been shown that both in vitro and in vivo chronic agonist treatment can lead to receptor up-regulation and, thus, enhanced radioligand internalization (41–43). On the basis of radioligand structure, comparative studies including a variety of radiometallated (111In and 64Cu) Phe3-octreotide, TOC, and TOCA derivatives, as well as radioiodinated sugar analogs of TOC and TOCA, revealed that ligand internalization is influenced by the nature of the chelate (DOTA) and can be substantially increased by Phe3-for-Tyr3 substitution and even more so by terminal Thr(ol)3-for-Thr8 exchange (5, 20, 21, 44). Furthermore, certain sugar residues were also shown to enhance ligand internalization. Whereas in many cases increased internalization is paralleled by an increased receptor affinity, octreotide analogs with comparable binding affinity were also found to exhibit considerable differences in internalization efficiency (44, 45). The same observations were made in the internalization studies performed with [123I]Gluc-S-Dpr(FP)TOCA, [123I]Gluc-S-Dpr(FBOA)TOCA, and [123I]Cel-S-Dpr(FBOA)TOCA (Table 3). In the case of [123I]Cel-S-Dpr(FBOA)TOCA and [123I]Gluc-S-Dpr(FBOA)TOCA, the increased internalization (i.e., the amount of internalized activity) of the cellobiose compared with the corresponding glucose analog is accompanied by a significantly higher EC50,R. That these two quantities correlate has already been demonstrated in a previous study with a series of glycosylated, radioiodinated TOC and TOCA analogs.6 In contrast to data obtained in the aforementioned study, however, the increased EC50,R of [123I]Cel-S-Dpr(FBOA)TOCA compared with its Gluc-counterpart also correlates with higher sst2- affinity (Table 2).

Interestingly, [123I]Gluc-S-Dpr(FP)TOCA shows an EC50,R value and a sst2 affinity nearly identical to that of [123I]Gluc-S-Dpr(FBOA)TOCA but a 1.4-fold enhanced internalization. The only structural difference between these two peptides consists in the size (FP < FBOA) and type (aliphatic versus aromatic) of the prosthetic group anchored to the Dpr3-side chain. In contrast to the significant improvement of receptor affinity by the transition from [123I]Gluc- to [123I]Cel-S-Dpr(FBOA)TOCA, replacement of the small FP group in [123I]Gluc-S-Dpr(FP)TOCA by the bulky aromatic FBOA group has no detectable influence on peptide receptor affinity, but it reduces ligand internalization. Therefore, it seems probable, that beside receptor affinity, ligand geometry also plays a significant role with respect to the internalization efficiency of radioiodinated octreotide derivatives.

Overall, radioligand accumulation inside tumor cells is not only determined by the extent of ligand internalization but also by the rates of externalization and subsequent reinternalization (recycling) and/or intracellular degradation of the radioligand. In externalization studies performed both under conditions allowing and inhibiting recycling (Fig. 3), the washout of [123I]Gluc-S-Dpr(FP)TOCA, [123I]Gluc-S-Dpr(FBOA)TOCA, and [123I]Cel-S-Dpr(FBOA)TOCA from tumor cells was found to be nearly identical and comparable with that of [123I]TOC. Thus, neither glycosylation nor the radiolabeling method have a detectable influence on externalization kinetics. These findings are in accordance with data from Köning et al. (46), which demonstrated agonist-independent rates of externalization for intact radioiodinated somatostatin analogs. The fact that for all of the fluorinated TOCA derivatives >96% of the initial cellular activity was externalized within 60 min under conditions inhibiting recycling (5 μM TOC) indicates a negligible extent of intracellular ligand residualization. This represents one major disadvantage of radiolabeled octreotide analogs, of which the charged radiometal-chelate-containing fragments remain trapped in the lysosomal compartments after degradation (7, 47, 48). The high extent of apparent intracellular retention of [123I]Gluc-S-Dpr(FP)TOCA and the two FBOA-analogs under conditions allowing recycling, however, demonstrate efficient reinternalization of externalized activity. This fact may contribute to the observed increase in in vivo tumor accumulation of all of the 18F-labeled TOCA derivatives in this study between 10 and 60 min p.i. despite the drastic reduction of blood activity levels and, thus, ligand availability during this time period. To improve “real” intracellular retention of radioiodinated peptides, how-
ever, the use of negatively charged prosthetic groups seems a promising approach (49).

In contrast to data from other studies (21), this study demonstrates that in vitro internalization as well as ECs0,R values only have limited predictive power with respect to in vivo tumor accumulation of the radioligands investigated. On the basis of the in vitro data, and assuming comparable biokinetics of the 18F-labeled peptides, similar and high tumor accumulation would have been expected for Gluc-S-Dpr([18]F)TOCA and Cel-S-Dpr([18]F)FBOA)TOCA, and a slightly lower accumulation of Gluc-S-Dpr([18]F)FBOA)TOCA. Unexpectedly, tumor uptake of the two [18]F)FBOA derivatives was very high and comparable at all of the time points investigated, whereas it was 35% lower in the case of Gluc-S-Dpr([18]F)FP)TOCA (Table 4). One possible explanation for this divergence, beside a major contribution of general ligand pharmacokinetics on in vivo tumor accumulation, may be the use of cells expressing the human sst2-receptor bearing an N-terminal hemagglutinin epitope tag for the in vitro experiments, whereas the biodistribution studies were performed using the rat pancreatic acinar tumor model AR42J. For [123 I]Gluc-TOCA, fundamentally different internalization characteristics and ECs0,R values were found when CHO (hsst2) and AR42J cells were used for the in vitro experiment, respectively. Because no significant effect of radioiodination of Tyr3 in TOC on sst2-affinity was observed (Table 2), it is unlikely that the use of the radioiodinated 18F-reference compounds instead of the 18F-labeled peptides in the internalization studies may have had an influence on the internalization characteristics of the respective radioligands in vitro.

That the accumulation of the four 18F-labeled TOCA analogs in tumor and other somatostatin receptor-positive tissues is predominantly receptor mediated was confirmed by in vivo competition studies (Figs. 5 and 6). Coinjection of an excess of unlabelled TOC led to a reduction of tumor uptake to 26–30% [Gluc-Lys([18]F)FP)TOCA and Gluc-S-Dpr([18]F)FBOA)-TOCA] and 18% [Gluc-S-Dpr([18]F)FP)TOCA and Cel-S-Dpr([18]F)FBOA)TOCA] of controls. In stomach and pancreas, ligand uptake was reduced to 30–60% and 38–48% of controls, respectively, for the peptides investigated. Generally, a comparably low dose of cold competitor was administered in this study (10–15 μg TOC per mouse) due to severe toxic side effects (cyanosis and swelling of paws and eyes in 50% of the cases followed by death in ≤30 min p.i.) observed in the Swiss nu/nu mice used, when higher doses were applied. This might explain the seemingly high extent of nonblockable accumulation of the 18F-labeled peptides in sst-expressing tissues. In other studies applying 50–100 μg of peptide per mouse, tumor uptake was reduced to a minimum of 7% (50) and 15% (21) of control. Interestingly, no significant extent of receptor-mediated ligand uptake was detected for any of the 18F-labeled TOCA derivatives in lung and adrenals, organs that have been shown to express somatostatin receptors (51, 52). These findings are in contradiction to results obtained with radioiodinated glucosylated TOC and TOCA analogs, as well as 64Cu-labeled triethylenetetramine-TOCA. In both studies, lung accumulation in nude mice was effectively blocked by coadministration of unlabeled competitor (21, 53).

Altogether, the applicability of an 18F-labeled octreotide analog for rapid and high-contrast PET imaging is not only determined by its absolute accumulation in target tissues, but also by the rate of elimination from the circulation and excretion organs and, thus, the achievable target:nontarget tissue ratios. It has been demonstrated for a series of glycosylated, radioiodinated TOC derivatives that the extent of hepatic and intestinal accumulation correlates with ligand lipophilicity (19). The same applies to the corresponding radioiodinated glucose-, maltose- and maltotriose-TOCA-analogs,4 [123]I]TOCA, [123]I]Gluc-TOCA, [123]I]Malt-TOCA, and [125]I]Mtr-TOCA, and also to the four 18F-labeled TOCA derivatives investigated in this study (Fig. 7). Interestingly, this correlation seems to be almost inde-

---

**Fig. 7** Correlation between the lipophilicity (log $P_{ow}$; $n = 6$) of glycosylated, radiohalogenated Tyr3-octreotate (TOCA) analogs and their activity accumulation in liver (left) and intestine (right) of AR42J tumorbearing nude mice 60 min p.i. (n = 3–5); bars, ±SD
dependent of the radiohalogenation or glycosylation method. Gluc-Lys\((^{18}\text{F})\text{FP}\)TOCA and Cel-S-Dpr\((^{18}\text{F})\text{FBOA}\)TOCA differ in both respects and, thus, in ligand geometry and net charge. Nevertheless, they have identical lipophilicities and, thus, show an identical biodistribution in the excetration organs 60 min p.i. (Table 4). Only in Gluc-S-Dpr\((^{18}\text{F})\text{FBOA}\)TOCA, the aromatic \((^{18}\text{F})\text{FBOA}\) group seems to structurally outweigh the sugar residue and, thus, leads to overproportional accumulation of this ligand in liver, intestine, and kidney.

It is generally assumed that charge-dependent endocytosis significantly contributes to the renal uptake of small bioactive peptides (54). The fact that kidney accumulation of radiometal-labeled octreotidic derivatives can be efficiently reduced by coinfusion of cationic amino acids during internal radiotherapy additionally supports this assumption (3, 55). A lower positive net charge of the peptide should, therefore, result in reduced kidney accumulation (56). This seems to be the case for Gluc-S-Dpr\((^{18}\text{F})\text{FP}\)TOCA in comparison with Gluc-Lys\((^{18}\text{F})\text{FP}\)TOCA: the loss of the positive charge on N\(_\text{e}\) of the N-terminal amino acid leads to a reduction in kidney accumulation to ~20% of the value found for the Amadori compound. On the other hand, net charge is also reduced in the case of the two \((^{18}\text{F})\text{FBOA}\) analogs, but renal activity levels remain almost unchanged compared with Gluc-Lys\((^{18}\text{F})\text{FP}\)TOCA. This observation suggests a significant additional “inverse” contribution of ligand geometry, in this case the presence of the bulky aromatic FBOA moiety, on kidney accumulation.

In conclusion, two of the \((^{18}\text{F})\) octreotides investigated in this study, Gluc-S-Dpr\((^{18}\text{F})\text{FP}\)TOCA and Cel-S-Dpr\((^{18}\text{F})\text{FBOA}\)TOCA, show excellent pharmacokinetic profiles optimal for in vivo PET imaging of sst-positive tumors. Their particularly high tumor:background ratios render them vastly superior to the most promising \((^{18}\text{F})\) octreotide analog developed thus far, Gluc-Lys\((^{18}\text{F})\text{FP}\)TOCA, of which the potency in high-contrast PET imaging has already been demonstrated in first patient studies. However, the inefficiency and impracticability of \((^{18}\text{F})\) fluorooacylation with respect to routine \((^{18}\text{F})\)-peptide labeling prevent application of Gluc-S-Dpr\((^{18}\text{F})\text{FP}\)TOCA in a clinical setting. In contrast, the development of the fast, chemoselective, and high-yield two-step methodology of peptide radiofluorination via oxime formation allows rapid preparation of high amounts of Cel-S-Dpr\((^{18}\text{F})\text{FBOA}\)TOCA, making this peptide, along with its excellent pharmacokinetics, a most promising tracer for routine PET somatostatin receptor imaging.

We assume that both the above radiohalogenation strategy based on chemoselective oxime formation as well as its combination with glycosylation using a trifunctional linker concept will provide access to a large variety of \(^{18}\text{F}, \text{123/125/131}\)I, or \(^{211}\text{At}\)-labeled bioactive peptides with optimized pharmacokinetics for in vivo receptor imaging as well as peptide receptor radiotherapy.

ACKNOWLEDGMENTS

We thank Claudia Bodenstein, Brigitte Dzewas, and Coletta Kruschke for excellent technical assistance. Furthermore, we thank Wolfgang Linke for supplying \(2-(^{18}\text{F})\text{fluoropropionic acid for the synthesis of the reference peptides.}

REFERENCES


First $^{18}$F-Labeled Tracer Suitable for Routine Clinical Imaging of sst Receptor-Expressing Tumors Using Positron Emission Tomography

Margret Schottelius, Thorsten Poethko, Michael Herz, et al.


Updated version  Access the most recent version of this article at:  
http://clincancerres.aacrjournals.org/content/10/11/3593

Cited articles  This article cites 53 articles, 10 of which you can access for free at:  
http://clincancerres.aacrjournals.org/content/10/11/3593.full#ref-list-1

Citing articles  This article has been cited by 15 HighWire-hosted articles. Access the articles at:  
http://clincancerres.aacrjournals.org/content/10/11/3593.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.