Supplementary material for Kiviniemi A, et al. Feasibility of experimental BT4C glioma models for somatostatin receptor 2-targeted therapies. Acta Oncol 2014;53:1125–34.

Supplementary Table I. Details of the antibodies and conditions used for the immunohistochemistry.

Antibody/clone	Source	Species samples	Dilution	Incubation	Method
SSTR2/UMB1	Abcam, Cambridge, UK	Rat	1:200	32 min at 37°C	Ventana Benchmark XT Autostainer LabVision autostainer with BrightVision detection kit (Immunologic, Duiven, the Netherlands)
		Mouse	1:100	60 min at RT	
CD34/QBend10	Ventana, Strasbourg, France	Rat	Ventana reagents	20 min	Ventana Benchmark XT Autostainer
CD68/PG-M1	Dako, Glostrup, Denmark	Rat	1:100	32 min at 37°C	Ventana Benchmark XT Autostainer

Supplementary Appendix

Material and methods

Preparation of [⁶⁸Ga]DOTATOC, [¹⁸F]FDR-NOC and [¹⁸F]FDG

The radiosynthesis of [⁶⁸Ga]DOTATOC was performed with fully automated synthesis device (Modular Lab, Eckert & Ziegler Eurotope GmbH, Berlin, Germany). ⁶⁸Ga was obtained from a ⁶⁸Ge/⁶⁸Ga generator (Eckert & Ziegler, Berlin, Germany) by eluting with 0.1 M HCl. Eluate was passed through a cation exchange cartridge to the reaction vial, containing 2 mL of 0.2 M sodium acetate buffer (pH 4.0) and DOTATOC (28 nmol, Bachem AG, Bubendorf, Switzerland). The reaction mixture was heated at 95°C and the crude product was purified by loading it onto C-18 cartridge. [⁶⁸Ga]DOTATOC was eluted with ethanol [1 mL, 50% (v/v)] into the end product vial. The product was formulated with saline (9 mg/mL). Radiochemical purity of the product was evaluated by high performance liquid chromatography (HPLC, LaChrom instruments, Hitachi; Merck Darmstadt, Germany; an online radioisotope detector Radiomatic 150 TR, Flow Scintillation Analyzer; Packard, Meriden, CT, USA). An analytical Jupiter C-18 column (5 μm, 300 Å, 150 × 4.6 mm, Phenomenex, Torrance, CA, USA) was used. A gradient with water (A) and acetonitrile (B), both containing 0.1% TFA, was used as follows: 82% A and 18% B at 0–2 min, 40% A and 60% B at 11–14 min, 82% A and 18% B at 15–20 min, flow rate 1 mL/min.

For [¹⁸F]FDR-NOC preparation, the conjugation reaction between the prosthetic group 5-deoxy-5-[¹⁸F]fluororibose and the peptide precursor NOC (0.3 mM, ABX GmbH, Radeberg, Germany) was carried out in anilinium buffer (0.3 M, pH 4.6) for 10 min at room temperature. The reaction mixture

was subjected to HPLC purification with a C-18 Jupiter Proteo column (250 × 10 mm, Phenomenex). [¹⁸F]FDR-NOC was formulated in phosphate-buffered saline (PBS) with a sterile filtration (0.22 μm) for injection. [¹⁸F]FDG was synthesized as described previously [1]. The radiochemical purity exceeded 95% and specific radioactivity was 0.3–1 TBq/μmol.

CD34 and CD68 Immunohistochemistry

In addition to sstr₂ immunohistochemistry, anti-human antibodies CD34 and CD68 were also tested on rat cryosections. CD34 antibody specifically detects endothelium. CD68 is a transmembrane glycoprotein expressed by macrophages, which are also known to express sstr₂. Other parenchymal cells were internal negative controls. Negative controls were also made by leaving out primary antibodies from staining protocols. Details of the antibodies used and conditions for the immunohistochemistry are presented in Supplementary Table I. The frozen slides were fixed in cold acetone for 5 min and the staining was either performed with the Ventana Benchmark XT Autostainer (Ventana Medical Systems, Strasbourg, France), Ventana Discovery XT Autostainer or with a LabVision Autostainer (Thermo Scientific Inc, Kalamazoo, MI, USA).

Western blot analysis

Western blot analysis was carried out to study sstr₂ expression in BT4C cell line using rat adrenal gland, kidney, and spleen as positive controls. Briefly, 100 mg of cells/tissues were homogenized in 400 μl of cold radio immuno precipitation assay (RIPA) buffer supplemented with protease inhibitors. The samples were centrifuged for 30 min at 13 000 rpm, and protein concentrations were determined by the standard Bradford assay in the supernatants. For each experiment, samples were normalized for

protein concentration and equal amounts of material in Laemmli sample buffer were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose filters. For immunoblotting, the anti-sstr₂ rabbit monoclonal antibody UMB-1 (Abcam, Cambridge, UK) was incubated overnight at 1:1000 dilution in bovine serum albumin (BSA)/Tris-buffered saline (TBS)/Tween 0.1%, followed by secondary antibody horseradish peroxidase (HRP)-conjugated swine anti-rabbit (Dako, Glostrup, Denmark). For the loading control the membranes were reprobed using 1:20.000 anti-Tubulin mouse polyclonal antibody in BSA/TBS/Tween 0.1% for 1 h, followed by secondary antibody HRP-conjugated rabbit anti-mouse (Dako, Glostrup, Denmark).

Supplementary Figure 1. BT4C glioma cells express sstr₂ by immunostaining. (A) Sstr₂ immunohistochemistry with UMB-1 antibody shows intense, mainly intracellular staining of BT4C glioma cells in rat brain. Endothelium served as an internal positive control (arrow head). (B) White matter of contralateral normal brain served as an internal negative control. (C) Immunostaining for CD68 shows only occasional macrophages in the rat BT4C glioma (arrow). CD68 is also expressed by endothelium. (D) Subcutaneous BT4C tumors in nude mice showed positive sstr₂ immunostaining but also areas of no sstr₂ expression. (E) Muscle served as negative control for sstr₂ immunostaining in mice. Bar = 100 μm.

Results

CD34 and CD68 Immunohistochemistry

Only few macrophage cells were observed in rat BT4C glioma when stained immunohistochemically with CD68 antibody (Supplementary Figure 1). CD68 was also expressed by endothelial cells. Immunohistochemical staining for CD34 confirmed prominent neovascularization in rat BT4C glioma (data not shown).

Reference

- [1] Hamacher K, Coenen HH, Stocklin G. Efficient stereospecific synthesis of no-carrier-added 2-[¹⁸F]-fluoro-2-deoxy-D-glucose using aminopolyether supported nucleophilic substitution. J Nucl Med 1986;27:235–8.

Supplementary Figure 2. BT4C glioma cells express sstr₂ by Western blot analysis. BT4C cell line and rat control tissue homogenates probed with UMB-1 antibody show sstr₂ expression.