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# Evaluation of $^{177}\text{Lu}$ -DOTA- $\text{sst}_2$ Antagonist Versus $^{177}\text{Lu}$ -DOTA- $\text{sst}_2$ Agonist Binding in Human Cancers In Vitro

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Somatostatin receptor targeting of neuroendocrine tumors using radiolabeled somatostatin agonists is today an established method to image and treat cancer patients. However, in a study using an animal tumor model, somatostatin receptor antagonists were shown to label  $\text{sst}_2$ - and  $\text{sst}_3$ -expressing tumors in vivo better than agonists, with comparable affinity even though they are not internalized into the tumor cell. In the present study, we evaluated the in vitro binding of the antagonist  $^{177}\text{Lu}$ -DOTA-pNO<sub>2</sub>-Phe-c (D-Cys-Tyr-D-Trp-Lys-Thr-Cys) D-TyrNH<sub>2</sub> ( $^{177}\text{Lu}$ -DOTA-BASS) or the  $^{177}\text{Lu}$ -DOTATATE agonist to  $\text{sst}_2$ -expressing human tumor samples. **Methods:** Forty-eight  $\text{sst}_2$ -positive human tumor tissue samples (9 ileal carcinoids, 10 pheochromocytomas, 7 breast carcinomas, 10 renal cell carcinomas, and 12 non-Hodgkin lymphomas) were analyzed by in vitro receptor autoradiography for the expression of  $\text{sst}_2$ , comparing the binding capacity of  $^{177}\text{Lu}$ -DOTA-BASS and  $^{177}\text{Lu}$ -DOTATATE in successive tissue sections. The autoradiograms were quantitated using an electronic autoradiography detection system. **Results:** In all cases, the radiolabeled antagonist bound to more receptor sites than did the agonist. The mean ratios of the antagonist  $^{177}\text{Lu}$ -DOTA-BASS to the agonist  $^{177}\text{Lu}$ -DOTATATE were  $4.2 \pm 0.5$  in the 9 ileal carcinoids,  $12 \pm 3$  in the 10 pheochromocytomas,  $11 \pm 4$  in the 7 breast carcinomas,  $5.1 \pm 0.6$  in the 10 renal cell carcinomas, and  $4.8 \pm 0.7$  in the 12 non-Hodgkin lymphomas. **Conclusion:** The present in vitro human data, together with previous in vivo animal tumor data, are strong arguments indicating that  $\text{sst}_2$  antagonists may be worth testing in vivo in patients in a wide range of tumors including nonneuroendocrine tumors.

**Key Words:** somatostatin receptors; cancer targeting; DOTA- $\text{sst}_2$  agonist; DOTA- $\text{sst}_2$  antagonist; receptor autoradiography

**J Nucl Med 2011; 52:1886–1890**

DOI: 10.2967/jnumed.111.095778

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**I**n the last few years, peptide hormone receptors have become increasingly attractive for diagnostic imaging and peptide-receptor radionuclide therapy because of their overexpression in certain human tumors (1–3). In particular,

somatostatin receptor tumor targeting currently constitutes a well-established method to image or to treat patients with somatostatin receptor-positive gastroenteropancreatic neuroendocrine tumors.  $^{111}\text{In}$ -pentetate (OctreoScan; Covi-dien), for instance, has been widely used to visualize these types of tumors (4,5). Furthermore, in clinical studies, somatostatin receptor radionuclide therapy with patients with neuroendocrine tumors has given promising results (6). Indeed, several excellent somatostatin agonists, among which are  $^{90}\text{Y}$ -DOTATOC and  $^{177}\text{Lu}$ -DOTATATE, have been developed in the last few years and are now routinely used in the clinic to treat patients with neuroendocrine tumors (7,8). An important feature of such agonist radioligands is that after binding to the receptor, they have to be internalized into the tumor cell by receptor-mediated endocytosis, leading to an accumulation of the radioactive agonist within the tumor cell (9,10). Interestingly, it has recently been shown for  $\text{sst}_2$  and  $\text{sst}_3$  in an animal tumor model that somatostatin antagonists with high affinity for the receptor can also have excellent properties for peptide receptor targeting (11). Despite the fact that there is a minimal internalization of the receptor-antagonist complex into tumor cells, the antagonist readily labels somatostatin receptor-expressing tumors at least as well as, and often even better than, agonists and has, moreover, a higher tumor uptake than the corresponding agonist, with comparable affinity. A reason for this observation may be that antagonists bind to a larger population of binding sites and have a lower dissociation rate than agonists (11). Confirming data were obtained with another peptide hormone receptor, the gastrin-releasing peptide receptor (12); this study showed, in comparative in vitro-in vivo experiments, the superiority of the antagonist demobesin 1 as a tumor-targeting agent to the comparable potent agonist demobesin 4. The aim of the present study was to evaluate the in vitro binding behavior of the antagonist  $^{177}\text{Lu}$ -DOTA-BASS, compared with the established agonist radioligand  $^{177}\text{Lu}$ -DOTATATE, on several different, well-characterized human tumor samples by in vitro receptor autoradiography.

## MATERIALS AND METHODS

### Reagents

All reagents were of the best grade available and were purchased from common suppliers. DOTA-pNO<sub>2</sub>-Phe-c (D-Cys-Tyr-D-Trp-Lys-Thr-Cys) D-TyrNH<sub>2</sub> (DOTA-BASS) and somatostatin-

Received Jul. 14, 2011; revision accepted Aug. 18, 2011.

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Published online Nov. 8, 2011.

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28 (SRIF-28) were provided by Jean E. Rivier (The Salk Institute), and DOTATATE was provided by Helmut R. Mäcke (Basel, Switzerland).

### Tissues

Fresh-frozen tissue samples from surgically resected tumors, which were characterized for their  $sst_2$  expression in previous studies (13–17), were used. The study conformed to the ethical guidelines of the Institute of Pathology of the University and the University Hospital of Berne and was reviewed by the Institutional Review Board.

### Preparation of Radiotracer $^{177}\text{Lu}$ -DOTA-BASS and $^{177}\text{Lu}$ -DOTATATE

$^{177}\text{Lu}$ -DOTA-BASS and  $^{177}\text{Lu}$ -DOTATATE were prepared after incubation of 5.5–6.5 nmol of each conjugate with 185–222 MBq of  $^{177}\text{LuCl}_3$  at 95°C for 30 min in 0.5 mL of sodium acetate buffer (0.4 M, pH 5.0). Quality control was performed by reversed-phase high-performance liquid chromatography as previously described (18). The radiotracer solutions were prepared by dilution with ascorbic acid solution (50 mg/mL, pH 5.0), in a radioactivity concentration of 37 MBq/mL (specific activity, 40 MBq/nmol).

### Binding Affinities Determined by Receptor Autoradiography Using [Leu<sup>8</sup>, D-Trp<sup>22</sup>, <sup>125</sup>I-Tyr<sup>25</sup>]-SRIF-28 (<sup>125</sup>I-LTT-SRIF-28)

Cell membrane pellets were prepared as previously described and stored at –80°C (19). Receptor autoradiography was performed on 20- $\mu\text{m}$ -thick cryostat (HM 500; Microm) sections of the membrane pellets, mounted on microscope slides, and then stored at –20°C as previously described (19,20). For each of the tested compounds, complete displacement experiments with the universal SRIF radioligand <sup>125</sup>I-LTT-SRIF-28 (74,000 GBq/mmol [2,000 Ci/mmol]; Anawa) using 15,000 cpm/100  $\mu\text{L}$  and increasing concentrations of the unlabeled peptide ranging from 0.1 to 1,000 nM were performed. As a control, unlabeled SRIF-28 was run in parallel, using the same increasing concentrations. The sections were incubated with <sup>125</sup>I-LTT-SRIF-28 for 2 h at room temperature in Tris-HCl buffer (170 mmol/L; pH 8.2), containing 1% bovine serum albumin (BSA), bacitracin (40 mg/L), and MgCl<sub>2</sub> (10 mmol/L) to inhibit endogenous proteases. The incubated sections were washed twice for 5 min in cold Tris-HCl (170 mmol/L; pH 8.2) containing 0.25% BSA. After a brief dip in Tris-HCl (170 mmol/L; pH 8.2), the sections were dried quickly and exposed for 1 wk to BioMax MR film (Kodak). Fifty percent inhibitory concentration values were calculated after quantification of the data using a computer-assisted image processing system as described previously (21). Tissue standards (autoradiographic <sup>125</sup>I or <sup>14</sup>C microscales; GE Healthcare) that contain known amounts of isotope, cross-calibrated to tissue-equivalent ligand concentrations, were used for quantification (20,22).

### In Vitro Receptor Autoradiography of Cancer Tissues with $^{177}\text{Lu}$ -Labeled Analogs

For in vitro somatostatin receptor autoradiography using  $^{177}\text{Lu}$ -labeled analogs, 20- $\mu\text{m}$ -thick cryostat (HM 500; Microm) sections of fresh-frozen tissue samples were mounted on microscope slides and then stored at –20°C as previously described (19,20). The sections were incubated with  $^{177}\text{Lu}$ -DOTATATE or  $^{177}\text{Lu}$ -DOTA-BASS using 20,000 cpm/100  $\mu\text{L}$  for 2 h at room temperature in Tris-HCl buffer (170 mmol/L; pH 8.2), containing 1% BSA, bacitracin (40 mg/L), and MgCl<sub>2</sub> (10 mmol/L) to inhibit endogenous

proteases. The incubated sections were washed twice for 5 min in cold Tris-HCl (170 mmol/L; pH 8.2) containing 0.25% BSA. Afterward, they were completely immersed for 10 s in Tris-HCl (170 mmol/L; pH 8.2) and then quickly dried. To determine the nonspecific binding of the  $^{177}\text{Lu}$ -labeled compounds, unlabeled DOTATATE or DOTA-BASS at a concentration of 100 nM was added to the radioactive samples and run in parallel. Radioactivity bound to the sections was quantitated immediately by electronic autoradiography (23). The electronic autoradiography detection system (InstantImager 2024; Packard Instruments) allowed a direct measurement of the radioactivity. The radioactive decays of the samples were measured for 60 min, and the counts per area were determined using the InstantImager quantitation software. The data obtained with the InstantImager were further validated using the following 2 different established methods: by exposition of the sections to BioMax MR film (Kodak) for 6, 17, or 66 h (depending on the receptor density on the tumor tissue), with subsequent quantification of the data for the determination of the binding affinities; and by counting the tissues for 1 min in a  $\gamma$ -counter (Wizard 1470 automatic  $\gamma$ -counter; PerkinElmer) after having carefully scraped the tissue material from the slide and transferred it into a counting vial. The 2 validation methods were found to give results comparable to those obtained with the InstantImager (not shown).

### Electronic Autoradiography

The InstantImager 2024 consists of a multiwire proportional chamber, filled with counting gas (argon, 96.5%; CO<sub>2</sub>, 2.5%; and isobutane, 1%), and a microchannel array detector.  $\beta$ -particles or secondary electrons from  $\gamma$ -radiation cause gas ionization in one of 214,420 microchannels of 0.4-mm diameter. The voltage potential in the channel causes an electron avalanche detected in the anode–cathode wire planes above. A fast digital processor calculates the position on the sample plate (20  $\times$  24 cm), and software running on a standard personal computer is used for tasks such as image acquisition and display. The online detection provides a direct quantitation of radioactivity in 2-dimensional samples. The electronic autoradiography system has been shown to be suitable for the use of most of the common radioisotopes used in nuclear medicine such as <sup>67</sup>Ga, <sup>99m</sup>Tc, <sup>111</sup>In, <sup>123</sup>I, <sup>125</sup>I, and <sup>131</sup>I (23). We have used the InstantImager to quantitate tissue cryosections from autoradiography experiments treated with compounds labeled with the  $\beta$ - and  $\gamma$ -emitter  $^{177}\text{Lu}$ . The data obtained by electronic autoradiography were validated by 2 additional methods (radiographic film and  $\gamma$ -counting), giving comparable results.

### RESULTS

Table 1 summarizes the  $sst_1$ – $sst_5$  affinity binding profile of the agonist DOTATATE and the antagonist DOTA-BASS obtained by in vitro receptor autoradiography using membrane pellet sections of transfected cells stably expressing the 5 somatostatin receptors. For comparison, the values of the natural SRIF-28 are shown as reference. Both compounds, DOTATATE and DOTA-BASS, exhibit a high selectivity for  $sst_2$ , and the 50% inhibitory concentration for  $sst_2$  is in the low nanomolar range and was identical for both compounds, a prerequisite to compare them directly in autoradiography binding experiments.

The radioligands  $^{177}\text{Lu}$ -DOTA-BASS and  $^{177}\text{Lu}$ -DOTATATE were furthermore evaluated for their ability to bind to

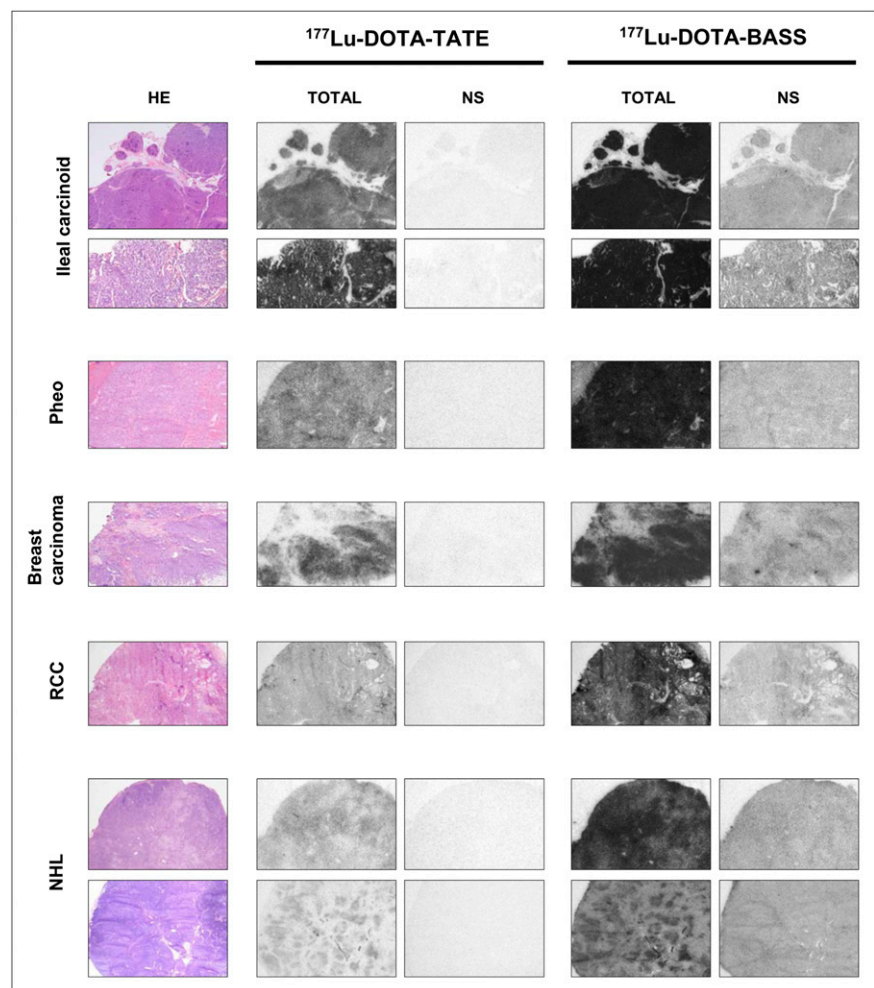
**TABLE 1**  
sst<sub>1</sub>–sst<sub>5</sub> Affinity Profile of 2 Somatostatin Analogs, Compared with SRIF-28 as Reference

Compound	sst <sub>1</sub>	sst <sub>2</sub>	sst <sub>3</sub>	sst <sub>4</sub>	sst <sub>5</sub>
SRIF-28, reference	3.2 ± 0.4 (7)	2.4 ± 0.2 (7)	4.0 ± 1.1 (7)	2.3 ± 0.5 (7)	2.3 ± 0.3 (7)
DOTATATE, agonist	>1,000 (3)	1.5 ± 0.4 (3)	>1,000 (3)	453 ± 179 (3)	547 ± 160 (3)
DOTA-BASS, antagonist	>1,000 (4)	1.5 ± 0.4 (4)	>1,000 (4)	287 ± 27 (4)	>1,000 (4)

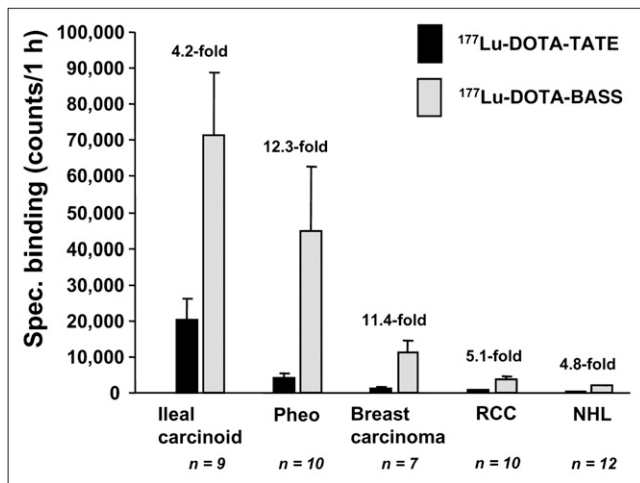
Data are expressed as 50% inhibitory concentration in nM (mean ± SEM).  
Number of experiments are in parentheses.

sst<sub>2</sub>-expressing tumor samples. To this end, receptor autoradiography was performed using 48 sst<sub>2</sub>-positive human tumor tissue samples (ileal carcinoid, pheochromocytoma, breast carcinoma, renal cell carcinoma, and non-Hodgkin lymphoma). Autoradiograms of representative examples for all 5 tumor types analyzed are shown in Figure 1. Both radioligands efficiently bind to the sst<sub>2</sub> receptor on the different tumor tissues (“total” column in Fig. 1), and competitive binding of the radioligands is observed after the addition of unlabeled DOTATATE and DOTA-BASS, respectively (“NS” column in Fig. 1). In all cases, the

sst<sub>2</sub>-expressing tumors were more intensely labeled in vitro with the antagonist <sup>177</sup>Lu-DOTA-BASS than with the agonist <sup>177</sup>Lu-DOTATATE. To obtain quantitative data, the autoradiography experiments were analyzed using the InstantImager as the electronic autoradiography detection system (23). Figure 2 shows bar graphs with the quantitation of the autoradiography experiments of the 5 different tumor types. The data illustrate the superiority of the antagonist <sup>177</sup>Lu-DOTA-BASS over the agonist <sup>177</sup>Lu-DOTATATE in respect to binding to the 5 tumor tissue types. The mean ratios of <sup>177</sup>Lu-DOTA-BASS to <sup>177</sup>Lu-DOTATATE were



**FIGURE 1.** Comparison of <sup>177</sup>Lu-DOTA-TATE and <sup>177</sup>Lu-DOTA-BASS receptor autoradiographic binding in successive sections of various types of human cancers (ileal carcinoid, pheochromocytoma, breast carcinoma, renal cell carcinoma, and non-Hodgkin lymphoma) expressing sst<sub>2</sub>. Columns from left to right represent hematoxylin and eosin staining, total and nonspecific binding of <sup>177</sup>Lu-DOTATATE, and total and nonspecific binding of <sup>177</sup>Lu-DOTA-BASS. Binding is markedly stronger with antagonist <sup>177</sup>Lu-DOTA-BASS. HE = hematoxylin and eosin; NHL = non-Hodgkin lymphoma; NS = nonspecific; Pheo = pheochromocytoma; RCC = renal cell carcinoma.



**FIGURE 2.** Quantitation of in vitro receptor autoradiography experiments with various types of human cancers (ileal carcinoid, pheochromocytoma, breast carcinoma, renal cell carcinoma, and non-Hodgkin lymphoma) using  $^{177}\text{Lu}$ -DOTATATE and  $^{177}\text{Lu}$ -DOTA-BASS as radioligands. Shown are bar graphs of specific binding (counts/h) of radioligands to tumor sections after quantitation using InstantImager. For all tested human tumor types, antagonist  $^{177}\text{Lu}$ -DOTA-BASS exhibited markedly better binding behavior. NHL = non-Hodgkin lymphoma; Pheo = pheochromocytoma; RCC = renal cell carcinoma; Spec. = specific.

$4.2 \pm 0.5$  in 9 ileal carcinoids,  $12 \pm 3$  in 10 pheochromocytomas,  $11 \pm 4$  in 7 breast carcinomas,  $5.1 \pm 0.6$  in 10 renal cell carcinomas, and  $4.8 \pm 0.7$  in 12 non-Hodgkin lymphomas. Additionally, the results obtained with the InstantImager were confirmed by 2 alternative detection methods: the autoradiography experiments were quantitated either by exposing the slides for an adequate time on radiographic films and determining the relative optical densities, or by transferring the labeled tissue from the slides to counting vials and measuring the cpm in a  $\gamma$ -counter (not shown).

## DISCUSSION

This in vitro study on human tumor tissues shows that antagonist radioligands bind to more  $\text{sst}_2$  receptors in tumor cells than agonist radioligands. It therefore complements a previous study performed in vivo in animal tumor models bearing  $\text{sst}_2$ - or  $\text{sst}_3$ -expressing tumors. In that study, Ginj et al. showed that radiolabeled somatostatin receptor antagonists are preferable to agonists for in vivo peptide receptor tumor targeting, leading to a change of paradigm in nuclear medicine (11).

We show here that the observation of more binding sites detected with antagonist radioligands can be made with a large variety of different tumor types. This observation can be made in the classic somatostatin receptor-expressing tumor targets such as ileal carcinoids, which can readily be targeted successfully in patients with agonist tracers (6). Our in vitro study suggests that the use of  $\text{sst}_2$  antagonist radioligands such as  $^{177}\text{Lu}$ -DOTA-BASS increases

tumor binding, compared with agonist binding, by 4.2 times, on average, in our 9 samples. This binding may increase the localization accuracy for tumors and metastases but also increase the efficacy of radiotherapeutic intervention with such antagonist tracers. The same observation and conclusions can be made for another type of neuroendocrine tumor, the pheochromocytoma, also often localized in patients with  $\text{sst}_2$  agonist radioligand (24); we found for this tumor an even higher increase in binding with the antagonist, namely 12.3-fold, compared with agonist.

Of particular interest are the results in the nonneuroendocrine, nonclassic tumor targets, namely tumors that normally express a low density of receptors, such as breast carcinomas, renal cell cancers, or non-Hodgkin lymphomas (14–16,25,26). Those tumors are not currently among those being routinely investigated with  $^{111}\text{In}$ -pentetreotide imaging. It is therefore impressive to see that by using the antagonist radioligand, it is possible to increase in vitro the number of binding sites in breast carcinomas and renal cell cancers 11.4- and 5.1-fold, respectively. Breast carcinomas and renal cell cancers reach, under such conditions, a receptor level much above (breast carcinomas) or almost equal to (renal cell cancers) the number of receptors detected in pheochromocytomas with the agonist radioligand. Our data therefore suggest that, using antagonist radioligands, compared with agonist imaging, it may be possible to also achieve in vivo an improved uptake relative to background in breast and renal cell cancers.

Non-Hodgkin lymphomas normally expressing a low amount of  $\text{sst}_2$  showed a 4.8-fold increase in receptor number with the antagonist radioligand, reaching levels that may be detected more easily in vivo than with current agonists (14,27). Thus, it is feasible that non-Hodgkin lymphomas might be amenable to radiotherapy with therapeutically radiolabeled antagonists, because these tumors are radiosensitive; they may therefore be more successfully treated with such a strategy than are currently the neuroendocrine tumors, known as not particularly radiosensitive tumors.

Once it is confirmed in a larger cohort of patients that classic neuroendocrine tumors can be better visualized with  $\text{sst}_2$  antagonists than with  $\text{sst}_2$  agonists, as recently demonstrated in a preliminary study with 5 patients (28), we may suggest, on the basis of the present study, that the in vivo targeting of nonclassic tumors such as breast carcinomas, renal cell cancers, and non-Hodgkin-lymphomas be reevaluated.

## CONCLUSION

The present in vitro human data, together with previous in vivo animal tumor data (11), are strong arguments indicating that  $\text{sst}_2$  antagonists may be worth testing in vivo in patients in a wide range of  $\text{sst}_2$ -expressing tumors, also including nonneuroendocrine tumors. Such  $\text{sst}_2$  antagonist radioligands should be useful not only for diagnostic tumor imaging but also for targeted tumor radiotherapy.

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