J. Endocrinol. Invest. *24:* 522-528, 2001

In vivo and *in vitro* expression of somatostatin receptors in two human thymomas with similar clinical presentation and different histological features

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ABSTRACT. [^{111}In-DTPA^{0}]octreotide scintigraphy allows the *in vivo* visualization of several types of SS receptor (SSR)-expressing tumors. Among these, thymomas have been recently detected. Here we report on 2 patients admitted for myasthenia gravis and radiological evidence of thymic mass. Although these patients had similar clinical presentation, *in vivo* SSR scintigraphy displayed a difference in the degree of the [^{111}In-DTPA^{0}]octreotide uptake. Considering that both thymic masses had comparable volume, [^{111}In-DTPA^{0}]octreotide level was significantly higher in one of the 2 tumors (tumor/background ratio of 5.7 *vs* 2.6). The SSR subtype expression pattern was studied *in vitro* on the surgically resected specimens by ligand binding techniques, quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry. According to the recent World Health Organization classification, the 2 tumors were classified A and B2 thymomas respectively. In membrane homogenates, we found a higher number of high affinity [^{125}I-Tyr^{11}]-SS-14 binding sites in the B2 thymomas (23.5±2.5 *vs* 12.0±0.4 fmol/mg membrane protein; $p<0.05$). RT-PCR analysis showed sst_1, sst_{2A} and sst_3 mRNA in the 2 thymoma tissues, whereas SS mRNA was detectable only in the A thymoma. Quantitative evaluation of RT-PCR data showed a comparable expression of the relative amount of sst_{2A} mRNA in both tumors, whereas a significant higher expression of sst_3 mRNA in the B2 thymoma. Sst_{2A} immunoreactivity was localized mainly on the endothelium of intratumoral vessels, whereas sst_3 was present on either tumoral epithelial cells or normal reactive thymocytes. The expression of sst_{2A} receptors in these tumors is in line with the *in vivo* visualization by [^{111}In-DTPA^{0}]octreotide, which is considered a sst_2-preferring ligand. However, since radioligand uptake was significantly higher in the B2 thymoma, which expressed the largest sst_3 mRNA levels, it might be possible that this subtype is involved in determining the tumor visualization during SSR scintigraphy. Apart from the affinity of the radioligand for the receptor, also the efficacy of the internalization of the radioligand-receptor complex might play a role in radioactivity accumulation during *in vivo* SSR scintigraphy. In fact, although octreotide binds with lower affinity to sst_3 receptors, this subtype displayed the highest amount of agonist-dependent receptor internalization compared to the other SSR subtypes. Moreover, sst_3 was localized on both tumor cells and reactive thymocytes, and these latter cells are characterized by a very active turnover of membrane molecules. Finally, although more cases need to be evaluated, the lack of detection of SS mRNA in the tumor presenting a more aggressive phenotype (B2 thymoma) might have physiopathological or prognostic significance.

(J. Endocrinol. Invest. *24:* 522-528, 2001)

Key-words: Thymoma, somatostatin receptor subtypes, somatostatin receptors scintigraphy, immunohistochemistry, reverse transcriptase polymerase chain reaction.

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Accepted March 29, 2001.

INTRODUCTION

SS receptors (SSR) have been demonstrated *in vivo* and *in vitro* in a large number of human neoplasms (1, 2). Human SSR-positive tumors show a high uptake of [^{111}In-DTPA^{0}]octreotide during *in vivo* scintigraphy, which has become a standard procedure in the work-

up of patients with neuroendocrine tumors to obtain an accurate estimation of disease spreading (2). Besides, SSR scintigraphy has been proposed to select candidates responsive to specific peptide treatment (1, 3). SSR scintigraphy has been successfully employed to visualize sites of disease in patients with abnormalities of the immune system (4, 5). Neoplastic and inflammatory lesions have been clearly visualized in lymphomas, granulomatous diseases and autoimmune diseases (4, 5). The *in vivo* evidence of SS binding opened new fields of interest for the role of SS and SSR in the immune system. Moreover, the presence of SSR has been demonstrated *in vitro* in lymphoid tissues and cells (4-6). A recent novel observation is the visualization of thymic epithelial tumors using [^{111}In-DTPA^{0}]octreotide (7-9). Although this seems to have relevance for selecting patients who may benefit from treatment with SS analogs (10), the real distribution of SS and its receptor subtypes in this rather unexplored category of tumors has not been accurately investigated so far. Moreover, considering the relatively low number of SSR expressed in these tumors and in lymphoid tissues in general, the contribution of different receptor subtypes in determining the binding of radiolabeled SS analogs has not yet been well established (4, 6, 9).

The present study starts from the *in vivo* evaluation by [^{111}In-DTPA^{0}]octreotide scintigraphy of SS-binding sites in 2 patients admitted for myasthenia gravis and radiological evidence of thymic mass. The surgically removed tissues were analyzed *in vitro* by classical binding studies, reverse transcriptase polymerase chain reaction (RT-PCR), and immunohistochemistry to unravel the expression and potential significance of SS and SSR subtypes in these thymic tumors. Using real time quantitative RT-PCR, we analyzed the relative amount of sst_{2A} and sst_3 mRNAs. By immunohistochemistry, which allows the localization of the receptor proteins at cellular level, we further investigated the cellular localization of sst_{2A} and sst_3 receptors in tumor sections.

PATIENTS AND METHODS

Patients and samples

Two patients (1 male, aged 35 yr, and 1 female, aged 55 yr) were admitted for myasthenia gravis associated with radiological evidence of mediastinal mass. Computed tomography (CT) scan confirmed the diagnosis of thymic enlargement. [^{111}In-DTPA^{0}]octreotide scintigraphy was performed as well. Thereafter, both patients underwent surgery. The removed tumors were histologically classified as A and B2 thymomas according to the World Health Organization (WHO) classification, corresponding to a benign thymoma of the clinicopathologic classification (medullary thymoma of the histological classification, A) and to a malignant thymoma (cortical thymoma of the histological classification, B2). Samples from these tumors were taken directly at operation, quickly frozen and stored at -80 C for ligand binding and RT-PCR studies. Additional samples were fixed in 10% paraformaldehyde for immunohistochemistry. The protocol was in accordance with the Helsinki Doctrine on Human Experimentation. Informed consent was obtained from the patients.

Somatostatin receptor scintigraphy

Scintigraphy with [^{111}In-DTPA^{0}]octreotide was performed as previously reported (9). Briefly, planar images were obtained 24 h after the injection of 228 Mbq [^{111}In-DTPA^{0}]octreotide (Mallinckrodt, Petten, The Netherlands). Imaging studies were performed using a two-headed gamma camera (Picker 2000, Picker Instruments, Cleveland, Ohio, USA) equipped with a medium-energy-collimator. Acquisition time for planar spot images was 15 min. Tumor/background ratio was calculated dividing the mean counts in the target region by the mean counts in a chest region nearby and corrected for the size of the region, as described elsewhere (9).

SSR binding studies

The method of membrane isolation and the reaction conditions were described elsewhere (11). Briefly, membrane preparations (30-50 µg protein) of tissue samples were incubated in a total volume of 100 µl at room temperature for 60 min with increasing concentrations of [^{125}I-Tyr^{11}]-SS-14 without and with excess (1 µM) of unlabeled SS-14 in HEPES buffer (10 mM HEPES, 5 mM $MgCl_2$ and 0.02 g/l bacitracin, pH 7.6) containing 0.2% BSA. After incubation, 1-ml ice-cold HEPES buffer was added to the reaction mixture, and membrane-bound radioactivity was separated from unbound by centrifugation during 2 min at 14,000 rpm in an Eppendorf microcentrifuge. The remaining pellet was washed twice in ice-cold HEPES buffer, and the final pellet was counted in a γ-counter (1470 Wizard, Wallac, Turku, Finland). Specific binding was taken to be total binding minus binding in the presence of 1 µM unlabeled ligands.

RT-PCR studies

RT-PCR was performed as previously described (9). Briefly, poly A^+ mRNA was isolated using Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway) from tissue samples. cDNA was synthesized using the poly A^+ mRNA captured on the Dynabeads Oligo $(dT)_{25}$ as solid phase and first strand primer. One-

tenth of the cDNA was used for each amplification by PCR using primer sets specific for human sst_{1-5}, SS, and hypoxanthine-guanine phosphoribosil transferase (HPRT). Several controls were included in the RT-PCR experiments. To ascertain that no detectable genomic DNA was present in the poly A^+ mRNA preparation (since the SSR subtype genes are intron-less), the cDNA reactions were also performed without reverse transcriptase and amplified with each primer-pair. Amplification of the cDNA samples with the HPRT-specific primers served as a positive control for the quality of the cDNA. To exclude contamination of the PCR reaction mixtures, the reactions were also performed in the absence of cDNA template in parallel with cDNA samples. As a positive control for the PCR reactions of SSR receptor subtypes, 0.001 to 0.1 µg of human genomic DNA, representing approximately 300 to 30,000 copies of sst-template, were amplified in parallel with the cDNA samples. As a positive control for the PCR of HPRT and SS, aliquots of a cDNA sample known to contain SS and HPRT mRNA were amplified, because these primer-pairs did enclose introns in the genomic DNA. To quantify sst_2 and sst_3 mRNAs, a quantitative RT-PCR was performed by TaqMan® Gold nuclease assay (The Perkin-Elmer Corporation, Foster City, CA) and the Abi Prism® 7700 Sequence Detection System (The Perkin-Elmer Corporation) for real-time amplification, according to the manufacturers instructions. Specific primer and probe sequences were used for the quantitative RT-PCR. The amount of sst_2 and sst_3 mRNA was determined by means of a standard curve generated in each experiment from known amounts of human genomic DNA. For the determination of the amount of HPRT mRNA, the standard curve was obtained by including dilutions of a pool of cDNAs known to contain HPRT. The amount of sst_2 and sst_3 mRNA was calculated relative to the amount of HPRT and is given in arbitrary units.

Immunohistochemical localization of sst_{2A} and sst_3 receptors

Immunohistochemistry was performed on 5-µm paraffin-embedded sections. The sections were deparaffinized, rehydrated, exposed to microwave heating (in citric acid buffer, pH 6.0) at 100 C for 15 min, rinsed in tap water followed by PBS. Thereafter, the sections were incubated for 15 min in normal goat serum (1:10 dilution in PBS+5% BSA), and then incubated with antibodies, raised in rabbit, against sst_{2A} (R2-88, gift from Dr. A. Schönbrunn) and sst_3 (Biotrend, Cologne, Germany) overnight at 4 C. The sst_{2A} and sst_3 antibodies were used at a dilution of 1:500 and 1:2000, respectively in phosphate buffered saline (PBS)+5% BSA. A standard streptavidin-biotinylated-alkaline phosphatase complex (ABC kit, Biogenix, San Ramon, CA, USA) was used according to the manufacturer's recommendation to visualize the bound antibodies. The sections were developed with New Fuchsine/Naphtol AS-MX, slightly counterstained with hematoxylin and mounted. Negative controls for immunohistochemistry included: 1) omission of the primary antibody; 2) preabsorbtion of the antibodies with the respective immunizing receptor peptides (at a concentration of 100 µM). A tissue was considered positive when the immunostaining was abolished by pre-absorption of the antibody with the respective peptide antigen.

Statistical analysis

Data are expressed as mean±SE. Binding experiments were performed at least twice. SSR binding data on membrane homogenates were analyzed by the method of Scatchard.

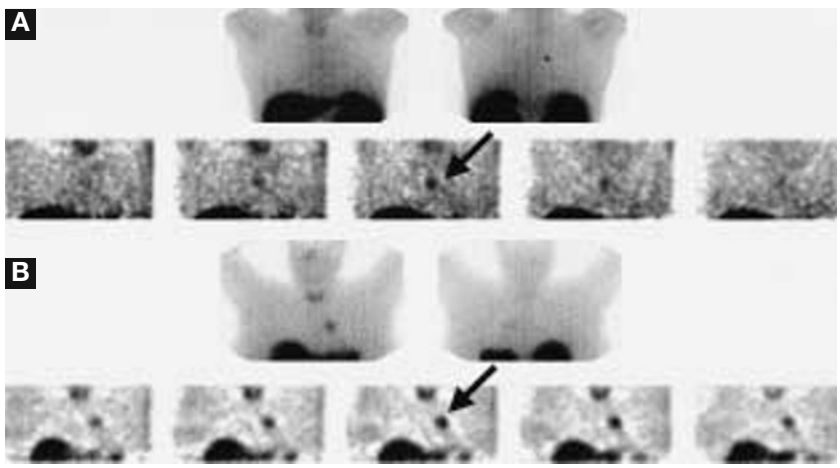

Fig. 1 - *Planar (top) and coronal SPECT (bottom) images of the chest 24 h after injection of [^{111}In-DTPA^0]octreotide. Planar anterior (left) and posterior (right) views. There is normal uptake in the thyroid and liver. An abnormal uptake in the region of the left hilum is seen (arrows). A) patient with A thymoma; B) patient with B2 thymoma.*

RESULTS

SSR binding studies

At SSR scintigraphy an abnormal uptake of [^{111}In-DTPA^{0}]octreotide was detected after 24 h in the mediastinum of both patients with thymoma. The uptake was significantly higher in the patient with the B2 thymoma compared to the patient with the A thymoma (Fig. 1). Tumor/background ratio calculated on SPECT reconstructions was 2.6 in the A thymoma and 5.7 in the B2 thymoma (Fig. 1).
Specific binding of [^{125}I-Tyr^{11}]-SS-14 was found on enriched membrane preparations of thymoma tissues. Scatchard analysis of the binding data showed an estimated K_d values of 0.4±0.1 and 0.5±0.2 nM, with a maximum binding capacity (B_{max}) of 12.0±0.4 and 23.5±2.5 fmol/mg membrane protein in the A and B2 thymomas, respectively ($p<0.05$ for B_{max} values).

RT-PCR studies

By RT-PCR, sst_1, sst_{2A} and sst_3 mRNA was found in the thymoma tissues, whereas sst_{2B}, sst_4 and sst_5 mRNA was undetectable. SS mRNA was expressed in the A thymoma, while it was undetectable in the B2 thymoma. Quantitative analysis of the sst_{2A} and sst_3 mRNA content showed a comparable expression of sst_{2A} mRNA in both tumor tissues (Fig. 2A). Conversely, the number of sst_3 mRNA copies was approximately 4-fold higher in the B thymoma compared to the A thymoma (Fig. 2A). This is also evident in Figure 2B, which shows the sst_3/sst_{2A} mRNA ratio.

Immunohistochemistry

By immunohistochemistry, sst_{2A} immunoreactivity was found on the endothelium of few small intratumoral vessels (Fig. 3A), while sst_3 immunoreactivity was observed on tumor cells and reactive thymocytes (Fig. 3B). In all cases, immunostaining could be completely abolished by pre-absorption with 100 nM of the respective peptide antigens (Fig. 3C and D).

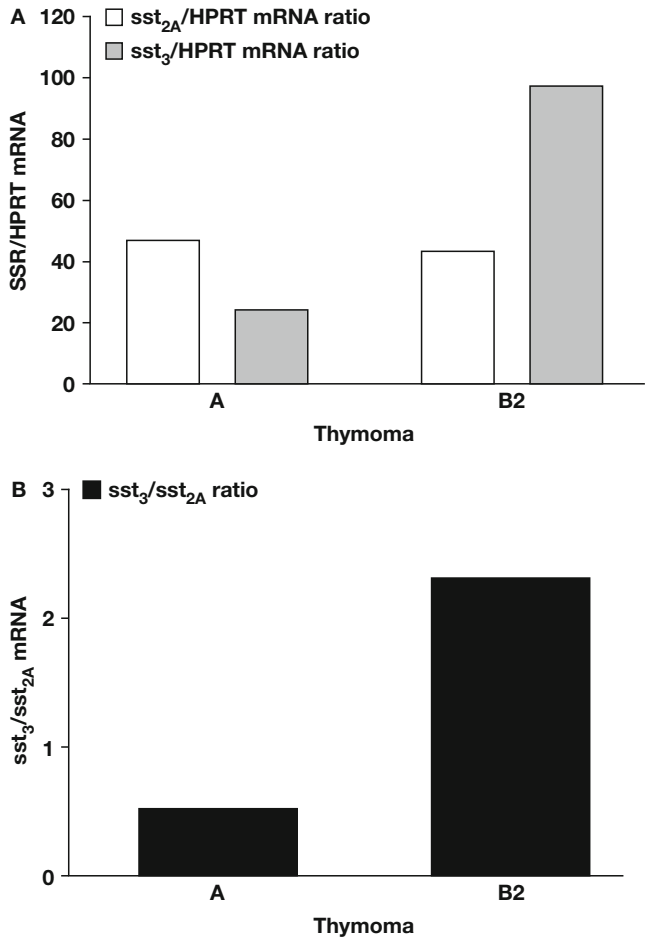

Fig. 2 - *Quantitative analysis of reverse transcriptase polymerase chain reaction (RT-PCR) data showing the different relative amount of sst_{2A} and sst_3 mRNAs in thymoma tissues calculated relative to the amount of hypoxanthine-guanine phosphoribosil transferase (HPRT) and given in arbitrary units.*

DISCUSSION

Thymoma is the most common tumor of the anterior-superior mediastinum. Metastases are rare, whereas locally aggressiveness, with infiltration of lungs, pleural and pericardial space, may occur (12). Thymomas are frequently associated with immunological disorders. Myasthenia gravis is the most common one, and occurs as typical complication of WHO type A, AB and B1-3 thymomas (12). Thymomas can be visualized by [^{111}In-DTPA^{0}]octreotide scintigraphy (7-10), currently used as diagnostic tool for neuroendocrine tumors, as well as experimentally for pathological entities involving the immune system (2, 4, 5). Moreover, therapy with SS analogs resulted in effective control of tumor growth in thymomas not responsive to conventional treatments (8, 10). We have recently demonstrated the *in vitro* expression of sst_1 sst_{2A} and sst_3 in the normal human thymus and in one thymoma (9, 11). Conversely, by autoradiography Reubi *et al.* failed to demonstrate SS-binding sites in 4 thymomas (6). To clarify the possible mechanisms involved in determining tumor uptake of [^{111}In-DTPA^{0}]octreotide, we investigated SSR expression in 2 human thymomas, analyzing and comparing the *in vivo* scintigraphic results to *in vitro* ligand binding, quantitative RT-PCR and immunohistochemical results. RT-PCR revealed a comparable expression of sst_{2A} and the absence of sst_5 in both cases, whereas a significantly higher sst_3 mRNA expression in the B2 thymoma was found. Indeed, this

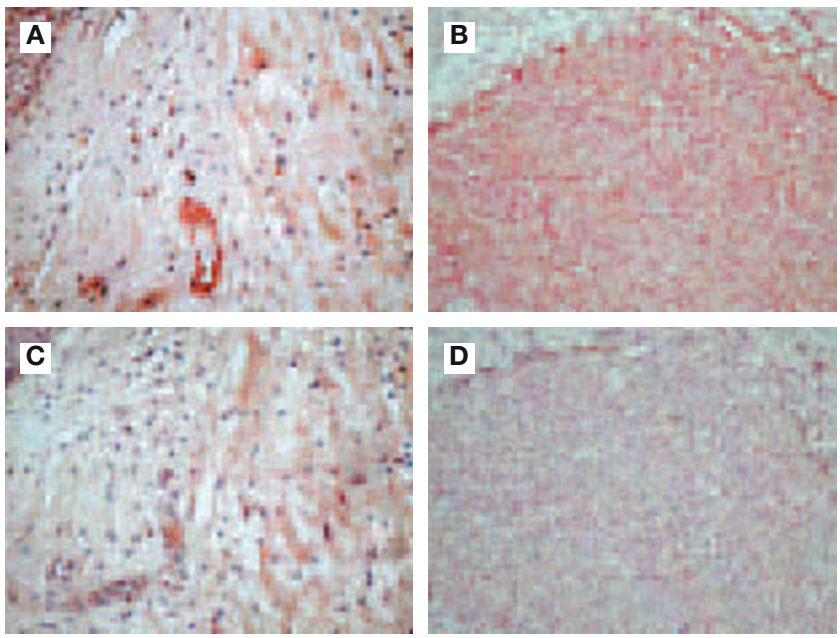

Fig. 3 - *Immunohistochemical detection of sst_{2A} and sst_3 receptors in paraffin-embedded sections from B2 thymoma. A) sst_{2A} immunoreactivity localized on the endothelium of an intratumoral vessel. B) sst_3 immunoreactivity within the thymocytes and tumor cells. C, D) Adjacent sections showing displacement of immunostaining after pre-absorption of the antibodies with 100 nM of the respective peptide antigens. Magnification 200 X. Sections developed with New Fuchsine/ Naphtol AS-MX. The sections are slightly counterstained with hematoxylin.*

latter tumor showed a higher number of SS-binding sites at membrane binding study, as well as a significant higher tumor/ background ratio compared to the A thymoma during *in vivo* SSR scintigraphy, suggesting that the involvement of sst_3 receptors in the uptake of [^{111}In-DTPA^0]octreotide cannot be ruled out. Radiolabeled octapeptides SS-analogs are internalized in a high amount by SSR-positive tumor cells (13, 14). [^{111}In-DTPA^0]octreotide is considered a sst_2-preferring ligand, suggesting a crucial role of sst_2 receptor in determining the accumulation of radioactivity following internalization of the radioligand-receptor complex. Evidence for the importance of sst_2 receptor derives from studies showing that sst_2-expressing cells internalize SS and octreotide. However, on the basis of the higher agonist-depending internalization rates of sst_3 compared to sst_2, it cannot be excluded that sst_3 receptor might play a role as well (15). Moreover, sst_3 has been detected by immunohistochemistry on both tumor cells and reactive thymocytes, whereas sst_{2A} receptors were found on endothelial cells only. Thymocytes are characterized by an active membrane molecule turnover. Indeed, B2 thymomas (also called cortical thymomas) contain the largest number of reactive thymocytes compared to the other tumor histotypes (12). The majority of human SSR-positive tumors that display a significant *in vivo* uptake of [^{111}In-DTPA^0]octreotide express multiple SSR subtypes (16). As further evidence, it has been recently shown that among several SSR scintigraphy-positive tumors, 3 thyroid tumors lacked the expression of sst_2 receptor (17), suggesting that tissue expression of sst_2 is not a prerequisite for positive imaging. Since the thyroid gland is constantly visualized during [^{111}In-DTPA^0]octreotide scintigraphy, an additional important observation is the lack or faint expression of sst_2 mRNA in normal thyroid tissue (17, 18). Although the presence of SS in the tumor does not seem to influence the outcome of *in vivo* SSR imaging, as it has been shown for medullary thyroid cancer (19), it cannot be fully excluded that the lack of SS mRNA expression in the B2 thymoma might play a role in the higher uptake of [^{111}In-DTPA^0]octreotide by this tumor as well.

The *in vivo* visualization of thymomas during SSR scintigraphy seems to be a rather complex phenomenon. First, *in vitro* binding studies showed that the number of SS-binding sites is significantly lower compared to neuroendocrine tissues and tumors. Second, the heterogeneity of receptor expression and perhaps additional regulatory mechanisms occurring *in vivo* may influence the affinity of a given receptor for radiolabeled SS-analogs and then the uptake of the tracer during SSR scintigraphy. It can be hypothesized that, while *in vitro* [^{125}I-Tyr^3]octreotide can be considered a rather "pure" sst_2-preferring ligand, *in vivo* the uptake of [^{111}In-DTPA^0]octreotide may occur in tissues or tumors expressing SSR with lower affinity for octapeptide SS analogs. Besides the role played in regulating radiolabeled SS-analog uptake, the significance of SSR expression

pattern in thymoma is intriguing, considering that an important aspect about these tumors has recently emerged. An original study demonstrated the presence of neuroendocrine differentiation in non-neuroendocrine thymic epithelial tumors (20). While "classic" neuroendocrine markers or cells are common features in thymic carcinoids, the presence of focal or dispersed neuroendocrine cells in thymic carcinoma and thymoma may reflect multidirectional differentiation within the tumor. The neuroendocrine differentiation may represent an additional marker of thymomas, next to cell atypia, expression of specific epithelial cell clusters and lack of immature T cell infiltration (20). Finally, the lack of detection of SS mRNA in the tumor displaying a more aggressive phenotype (B2 thymoma) might also have physiopathological or prognostic significance.

Further data on the internalization of SSR ligands by cells endogenously expressing SSR are mandatory in order to elucidate several remaining questions regarding the role of the different subtypes. In fact, apart from [^{111}In-DTPA^{0}]octreotide, other radiolabeled SS analogs suitable for diagnostic or therapeutic applications have been synthesized (21, 22). Moreover, radiotherapy and targeted chemotherapy using SS analogs is considered a future effective and feasible approach to treat patients with advanced metastatic SSR-positive tumors (23, 24).

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