0163-769X/03/$20.00/0
*Printed in U.S.A.*

Endocrine Reviews 24(1):28–47
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doi: 10.1210/er.2000-0001

# The Pathophysiological Consequences of Somatostatin Receptor Internalization and Resistance

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**Somatostatin receptors expressed on tumor cells form the rationale for somatostatin analog treatment of patients with somatostatin receptor-positive neuroendocrine tumors. Nevertheless, although somatostatin analogs effectively control hormonal hypersecretion by GH-secreting pituitary adenomas, islet cell tumors, and carcinoid tumors, significant differences are observed among patients with respect to the efficacy of treatment. This may be related to a differential expression of somatostatin receptor subtypes among tumors. In addition, the property of somatostatin receptor subtypes to undergo agonist-induced internalization has important consequences for visualizing, as well as for therapy, of receptor-postive tumors using radioisotope- or chemotherapeutic-compound-coupled somatostatin analogs. This review covers the pathophysiological role of somatostatin receptor subtypes in determining the efficacy of treatment of patients with somatostatin receptor-positive tumors using somatostatin analogs, as well as the preclinical and clinical consequences of agonist-induced receptor internalization for somatostatin receptor-targeted radio- and chemotherapy. Herein, the development and potential role of novel somatostatin analogs is discussed. (*Endocrine Reviews* 24: 28–47, 2003)**

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## I. General Introduction

SINCE ITS DISCOVERY in 1973 by Guillemin and Gerich (1), knowledge of the functional role of somatostatin (SS) in regulating neurotransmission in the brain, as well as in the regulation of secretion processes in the anterior pituitary gland, the pancreas, and the gastrointestinal tract, has increased considerably. In addition to playing an important regulatory role in neurotransmission and secretion, the peptide may control cell proliferation in normal and tumorous tissues as well (2, 3). Between 1992 and 1994, five SS receptor (sst) subtype genes were cloned and characterized; they were code-named $sst_1$, $sst_2$, $sst_3$, $sst_4$, and $sst_5$ (4). The discovery of these genes initiated a large number of studies directed to elucidate their expression in SS-target tissues, their selectivity of binding of structural SS-analogs, and their coupling to the different second messenger systems known to be activated upon SS binding to its receptor. This has been reviewed extensively (5–9). The discovery of the sst subtype genes also initiated the development of a large series of novel SS-analogs that selectively bind to sst subtypes. Currently, a number of these sst subtype-selective analogs are being tested for their *in vivo* and *in vitro* potencies to modulate hormone secretion and/or cell proliferation (8, 10). The high density of SS receptors on human neuroendocrine tumors originating from normal SS target tissues has been used clinically to treat symptoms of hormonal hypersecretion in patients with GH- or TSH-secreting pituitary adenomas, as well as in patients harboring islet cell or carcinoid tumors with SS-analogs (11). However, although SS-analogs effectively control hormonal hypersecretion by neuroendocrine tumors, their effects are often transient, and considerable differences between patients harboring islet cell and carcinoid tumors exist with respect to the development of tachyphylaxis. In addition, the presence of a high density of SS receptors on human neuroendocrine tumors has allowed the development in 1989 (2, 12) of the technique of sst scintigraphy using radiolabeled SS-analogs to visualize sst-positive tumors *in vivo* (2, 13).

The above physiological and pathophysiological roles of SS and the presence of SS receptors on neuroendocrine tumors have been reviewed extensively. Much less attention has been paid to the clinical importance of sst internalization in determining the uptake of radiolabeled SS-analogs by sst-positive neuroendocrine tumors and the role of individual sst subtypes herein, as well as to the mechanisms in-

Abbreviations: CHO, Chinese hamster ovary; DOTA, tetraazacyclododecane tetraacetic acid; DTPA, diethylenetriamine pentaacetic acid; hsst, human sst; 5-HIAA, 5-hydroxyindolacetic acid; $^{177}$Lu, $^{177}$lutetium; MTC, medullary thyroid carcinoma; PRL, prolactin; SS, somatostatin; sst, SS receptor; wt, wild-type; $^{90}$Y, yttrium-90.



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volved in tachyphylaxis to SS-analog therapy. This manuscript gives an overview of the current knowledge on the internalization and cellular uptake of radiolabeled SS-analogs by sst-positive tumor cells and the involvement of endogenously expressed sst subtypes in this process, as well as the clinical consequences of sst internalization for sst-targeted radio- or chemotherapy. *Section III* of this review addresses the potential mechanisms involved in tachyphylaxis after long-term treatment of patients with neuroendocrine tumors with SS-analogs.

### *A. SS and sst subtypes*

SS is a small cyclic peptide that is widely expressed throughout the central nervous system and peripheral tissues. In peripheral tissues, SS exerts predominantly inhibitory actions (14) on secretion processes, whereas the peptide acts as a neurotransmitter in both a stimulatory and inhibitory manner in the brain (15). SS is formed by proteolytic processing of larger precursor molecules, *i.e.*, prepro-SS and pro-SS. After cleavage of the pro-SS molecule, two biologically active forms of SS consisting of 14 (SS-14) or 28 (SS-28) amino acids are generated (16). SS-14 and SS-28 act via high-affinity G protein-coupled membrane receptors. Five sst subtypes have been cloned and characterized. The genes encoding the five sst subtypes are localized on different chromosomes (8). Via alternative splicing, two forms of the $sst_2$ receptor can be generated, *i.e.*, $sst_{2A}$ and $sst_{2B}$ (17, 18). The only difference between $sst_{2A}$ and $sst_{2B}$ is the length of their cytoplasmic tail. The five sst subtypes share a coupling to the second messenger systems known to be activated upon SS binding to its receptor. These systems include inhibition of adenylyl cyclase activity and activity of calcium channels, as well as stimulation of phosphotyrosine phosphatase or MAPK activity. This has been reviewed extensively (7–9). Although the inhibitory effects on adenylate cyclase activity and on the influx of $Ca^{2+}$ are linked to inhibition of secretion processes, the activation of phosphotyrosine phosphatase or MAPK activity may play a role in the regulatory effects that SS exerts on cell proliferation (2, 3, 10). The selective induction of apoptosis mediated via activation of $sst_3$ receptors is of particular interest in this respect. The role of the individual sst subtypes and the mechanism of action of the antiproliferative effects by SS have been reviewed recently (9). The five sst subtypes all bind SS-14 and SS-28 with high affinity but can be divided into two subclasses on their ability to bind structural octapeptide analogs of SS. The $sst_1$ and $sst_4$ receptors do not bind octapeptide SS-analogs, whereas $sst_{2A}$, $sst_3$, and $sst_5$ receptors display a high, low, and moderate affinity, respectively, toward octapeptide SS-analogs such as the clinically used octreotide and lanreotide (Table 1).

### *B. SS receptor subtype expression in normal and tumorous human tissues*

Classical SS-target tissues such as the central nervous system, the anterior pituitary gland, and the pancreas express multiple sst subtypes. The expression of sst subtypes in the brain has only been studied extensively in rodent species. In the brain, mRNAs encoding for all five sst subtypes are expressed in a highly specific pattern (8). This regional, characteristic expression pattern of sst subtypes in the brain has recently been confirmed at the protein level by immunohistochemical techniques using sst subtype-specific antibodies (19). The adult human pituitary gland expresses $sst_1$, $sst_2$, $sst_3$, and $sst_5$ mRNAs, but not $sst_4$ mRNA (20). In addition, human pancreatic islet cells express all five sst subtype proteins, as determined by immunohistochemistry (21, 22). In human islets, $sst_1$, $sst_2$, and $sst_5$ receptors are the most abundantly expressed subtypes, with a high percentage of $\beta$-cells expressing $sst_1$ and $sst_5$, $\alpha$-cells expressing $sst_2$, and $\delta$-cells expressing $sst_5$ (22).

TABLE 1. SS receptor subtype selectivity of binding of SS agonists

| Agonist | Binding potency ($IC_{50}$ values in nM) | | | | |
|---|---|---|---|---|---|
| | $sst_1$ | $sst_2$ | $sst_3$ | $sst_4$ | $sst_5$ |
| SS-14 | 2.3 | 0.2 | 1.4 | 1.8 | 0.9 |
| Octreotide | >1000 | 0.6 | 34.5 | >1000 | 7 |
| Lanreotide | >1000 | 0.8 | 107 | >1000 | 5.2 |
| Vapreotide[a] | >1000 | 5.4 | 31 | 45 | 0.7 |
| MK-678[a] | >1000 | 1.5 | 27 | 127 | 2 |
| BIM-23268 | 18.4 | 15.1 | 61.6 | 16.3 | 0.4 |
| BIM-23197 | >1000 | 0.2 | 26.8 | >1000 | 9.8 |
| BIM-23244 | >1000 | 0.3 | 133 | >1000 | 0.7 |
| SOM-230 | 9.3 | 1.0 | 1.5 | >100 | 0.2 |

Data are derived from Refs. 8, 74, and 191.
[a] Values represent $K_i$ values in nM (Refs. 8, 187, 188, 191).

Neuroendocrine tumors, which often originate from SS-target tissues, frequently express a high density of SS receptors (23–26). The sst-expressing human tumors include pituitary adenomas, islet cell tumors, carcinoids, paragangliomas, pheochromocytomas, small cell lung cancers, and medullary thyroid carcinomas (MTCs), but also breast cancers and malignant lymphomas (24, 27). The sst subtype expression in different types of human cancers has been demonstrated at the mRNA level using *in situ* hybridization (28, 29), RNase protection assays, and RT-PCR (20, 27, 30–34). The majority of human sst-positive tumors simultaneously express multiple sst subtypes, although there is a considerable variation in sst subtype expression between the different tumor types and among tumors of the same type. Table 2 shows that $sst_2$ is the most abundantly expressed receptor subtype in the majority of tumors. Recent studies using antibodies raised against synthetic peptide sequences of the $sst_1$, $sst_2$, $sst_3$, and $sst_5$ receptor confirmed this variation in the expression of sst subtypes in different types of human tumors (Table 2; Refs. 35–38). The higher number of tumors expressing particular sst subtype mRNAs, compared with the number of tumors expressing sst subtype proteins, may be related to the higher sensitivity of techniques such as RT-PCR compared with immunohistochemistry. On the other hand, techniques such as RT-PCR might overestimate the real percentage of tumors expressing sst subtypes because blood vessels, immune cells, stromal and contaminating normal cells, which are present in or surround human tumors, may express sst subtypes as well (29, 39–41). The predominant expression of $sst_2$ receptors in human tumors forms the basis for the successful clinical application of octapeptide SS-analogs such as octreotide and lanreotide in controlling symptoms related to hormonal hypersecretion in patients with GH-secreting pituitary adenomas, islet cell tu-

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TABLE 2. Expression of sst subtypes in human tumors

| Tumor type | SS receptor subtype | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $sst_1$ | | $sst_2$ | | $sst_3$ | | $sst_4$ | | $sst_5$ | |
| | mRNA | Protein | mRNA | Protein | mRNA | Protein | mRNA | Protein | mRNA | Protein |
| Pituitary tumor | | | | | | | | | | |
| Somatotroph | 44% (25) | | 96% (28) | | 44% (25) | | 5% (22) | | 86% (22) | |
| Lactotroph | 84% (19) | | 63% (19) | | 35% (17) | | 6% (17) | | 71% (17) | |
| Nonfunctioning | 38% (24) | | 75% (24) | | 43% (23) | | 13% (23) | | 48% (23) | |
| Corticotroph | 56% (9) | | 67% (9) | | 25% (8) | | 0% (7) | | 86% (7) | |
| Neuroendocrine GEP tumors | | | | | | | | | | |
| Carcinoid | 76% (59) | 88% (8) | 80% (84) | 78% (63) | 43% (58) | | 68% (47) | | 77% (44) | |
| Gastrinoma | 79% (28) | 100% (5) | 93% (28) | 100% (8) | 36% (28) | | 61% (23) | | 93% (28) | |
| Insulinoma | 76% (21) | | 81% (21) | | 38% (21) | | 58% (19) | | 57% (21) | |
| Nonfunctioning ICT | 58% (24) | | 88% (24) | | 42% (24) | | 48% (21) | | 50% (24) | |
| Renal cell cancer | 85% (13) | | 100% (13) | | 0% (13) | | 50% (12) | | | |
| Breast cancer | 33% (103) | 52% (33) | 99% (103) | 48% (33) | 38% (101) | 48% (33) | 23% (97) | | 18% (51) | |
| Meningioma | 46% (24) | | 100% (24) | | 33% (24) | | 50% (12) | | 71% (14) | |
| Glioma | 100% (7) | | 100% (7) | | 67% (6) | | 71% (7) | | 57% (7) | |
| Neuroblastoma | 0% (6) | | 100% (15) | | 17% (6) | | | | | |
| Colorectal cancer | 27% (41) | | 87% (41) | | 22% (41) | | 10% (41) | | 46% (41) | |
| MTC | 29% (14) | | 79% (14) | | 36% (14) | | 0% (14) | | 64% (14) | |
| Pheochromocytoma | 100% (11) | 80% (5) | 100% (11) | 90% (20) | 73% (11) | | 73% (11) | | 73% (11) | |

Data on mRNA expression are derived from Refs. 20, 27, 30–34, 69, 70, 72, 192–199 and include studies using RT-PCR, Northern blotting, and *in situ* hybridization techniques. Data on protein expression include immunohistochemical studies using sst subtype-specific antibodies and are derived from Refs. 36–38 and 69. The values represent the percentage of tumors expressing the sst subtype among the tumors screened; the values between *parentheses* indicate the total number of tumors of the studies included. GEP, Gastroenteropancreatic; ICT, islet cell tumor; MTC, medullary thyroid carcinoma.

mors, or carcinoid tumors (2, 11), but also for the possibility to visualize sst-positive tumors using radiolabeled SS-analogs (see *Section II*). Knowledge of the sst subtype expression patterns in human neuroendocrine tumors may be very important for the development of the concept of sst-targeted radiotherapy or chemotherapy. As will be discussed below, sst subtypes differ in their ability to internalize receptor-bound ligand, which is a crucial step to direct a SS-analog-linked radioisotope or cytotoxic compound to the nucleus of the tumor cell.

### C. Agonist-induced internalization of sst subtypes

Since the cloning of the five sst subtypes, the involvement of the individual sst subtypes in the process of receptor-mediated internalization of SS has been extensively investigated. Although differences have been reported between human and rat sst subtypes with respect to their dynamics of agonist-induced internalization, *Section I.C* is focused primarily on human sst subtypes and briefly summarizes their reported ability to undergo internalization after exposure to agonists. The mechanisms involved in receptor-mediated internalization of sst subtypes are not the focus of this review, and they have been reviewed elsewhere (8, 9, 42–46). In general, the mechanism and route of internalization of sst-agonist complexes follow those described for many other G protein-coupled receptors (47–50) and involve aggregation of the hormone receptor complex in specialized areas of the membrane, followed by internalization of the hormone-receptor complex via clathrin-coated, as well as uncoated, pits (47, 51). After internalization and pit formation, fusion of these vesicles with lysosomes occurs, resulting in hormone degradation or receptor recycling to the cell surface (Fig. 1; Refs. 49, 52, and 53).

The sst subtypes differentially internalize SS and SS-analogs (9). In Chinese hamster ovary (CHO)-K1 cells stably expressing one of the five human sst subtypes, $sst_2$, $sst_3$, $sst_4$, and $sst_5$ receptors displayed rapid (within minutes) agonist-dependent internalization of [$^{125}$I]LTT SS-28 ligand in a time- and temperature-dependent manner (54). Maximum internalization of the radioligand occurred within 60 min. The $sst_3$- and $sst_5$-expressing cells displayed the highest degree of internalization (78% and 66%, respectively), followed by $sst_4$ (29%) and $sst_2$ (20%). In contrast, the $sst_1$ subtype displayed only a very low amount (4%) of internalization. Another study using COS-7 cells transfected with the human $sst_1$ ($hsst_1$) or $hsst_{2A}$ receptor subtypes (55) recently confirmed the low internalization rate of the $sst_1$ subtype. These investigators used confocal microscopy to analyze the fate of internalized novel fluorescent SS derivatives (43). In cells transfected with $sst_{2A}$ receptors, up to 75% of specifically bound fluorescent ligand was recovered inside the cells within 60 min after agonist exposure, where it clustered into small endosome-like particles (55), whereas the capacity of internalization of SS via the $sst_5$ receptor was intermediate between $sst_1$ and $sst_{2A}$ receptors (43). These particles increased in size over time, suggesting that the receptor-ligand complexes followed an endocytotic pathway.

Recent observations by Rocheville *et al.* (56) demonstrated that internalization of human sst subtypes can be determined by functional homo- and heterodimerization of sst subtypes as well. The $hsst_1$ receptors displayed no internalization of their selective ligand $^{125}$I-SCH288, consistent with their inability to undergo agonist-induced internalization as a monotransfectant (57). However, when $hsst_1$ receptors were cotransfected with a c-tail deletion mutant of $hsst_5$, a slight but significant internalization of $^{125}$I-SCH288 at 60 min was

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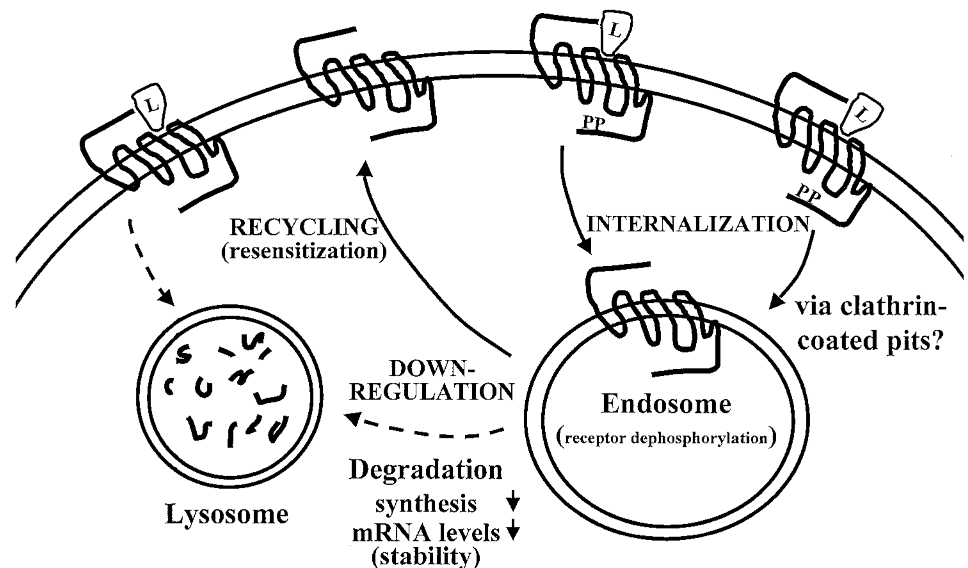


FIG. 1. Schematic representation of intracellular routing of G protein-coupled receptors (GPCRs) after agonist activation. After agonist activation, GPCRs are phosphorylated (involving protein kinase A, protein kinase C, and GPCR kinases) and internalized, probably via the formation of clathrin-coated pits (involving β-arrestins). The internalized receptors are then directed to endosomes in which they are dephosphorylated. Subsequently, the receptors are recycled back to the plasma membrane as functional (resensitized) receptors. GPCR down-regulation results from lysozomal degradation of intracellular receptors, decreased mRNA and receptor protein synthesis, as well as increased degradation via mobilization of membrane receptors directly to the lysosomal compartment. L, Ligand; PP, phosphate group. [Adapted from Ref. 49.]

observed. Heterodimerization of epitope-tagged $sst_{2A}$ and $sst_3$ receptors prevents agonist-induced endocytosis of $sst_3$ but not $sst_{2A}$ receptors (58). Apart from changes in functionality of individual sst subtypes due to receptor dimerization, sst receptors may also form heterodimers with other G protein-coupled receptors, *e.g.*, dopamine and opioid receptors (59, 60). Again, such heterodimers have properties different from the individual receptors. Therefore, the knowledge that homo- and heterodimerization of sst subtypes, and of sst subtypes with other G protein-coupled receptors, may influence the capacity of individual sst subtypes to undergo agonist-induced endocytosis clearly indicates the need to study internalization of sst subtypes endogenously expressed in sst-positive cells. Such studies will help to clarify the apparent discrepancies in internalization of specific sst subtypes. The above-described ability of sst subtypes to undergo agonist-induced internalization is an important characteristic of these receptors for transporting radiolabeled SS-analogs into the cell, thereby making sst-targeted radiotherapy a feasible approach to treat patients with neuroendocrine tumors expressing a high density of sst. In *Section II*, the preclinical evidence for internalization of radiolabeled SS-analogs, resulting in accumulation of intracellular radioactivity, by tumor cells endogenously expressing sst subtypes, is reviewed.

## II. Consequences of sst Internalization for sst-Targeted Radiotherapy or Chemotherapy of sst-Positive Tumors

### *A. Preclinical evidence for internalization of radiolabeled SS-analogs*

Human sst-positive tumors show a high uptake of [$^{111}$In-DTPA$^0$]octreotide at sst scintigraphy (13). Analysis of the uptake of [$^{111}$In-DTPA$^0$]octreotide by scintigraphy is preferably performed 24 h after the injection of the radiopharmaceutical (13). After 24 h, it is unlikely that the radioactivity in the tumors reflects cell membrane-bound ligand, but in fact, more likely, represents internalized radioligand. Internalization of [$^{111}$In-DTPA$^0$]octreotide *in vivo* is also evidenced by our observations in rats, in which uptake of radioactivity in sst-positive organs, such as the pituitary gland and the pancreas, after the injection of the radiopharmaceutical, can be prevented by injection of excess unlabeled octreotide up to 10 min post injection, but not 20 min post injection. At that time, all radioactivity present in the sst-positive tissues probably reflects internalized radioligand (61). Direct evidence for internalization and subsequent subcellular distribution of radioisotopes delivered to the tumor cells using radiolabeled SS-analogs is presented from several *ex vivo* and *in vitro* autoradiographic studies. After incubation of human HT-29 colon carcinoma cells with the $^3$H-labeled SS-analog TT-232, radioactivity was observed at the cell surface and cytoplasmic membranes, as well as the nucleus (62). Comparable observations were made in primary cultures of human carcinoid and gastrinoma cells incubated *in vitro* with [$^{111}$In-DTPA$^0$]octreotide (63). The primary cultures specifically bound and internalized this radiopharmaceutical. About 50% of the internalized radioactivity was released by the cells within 6 h, whereas the remaining radioactivity was trapped within the cells up to 42 h. Electron microscopic autoradiography demonstrated the presence of the internalized $^{111}$In in the cytoplasm and the nucleus. The same processes also apply to the *in vivo* situation. From seven patients with malignant midgut carcinoid tumors, who received an iv injection of 200 MBq [$^{111}$In-DTPA$^0$]octreotide 2 d before abdominal surgery, tumor tissue was obtained and analyzed for the subcellular distribution of radioactivity using ultrastructural autoradiography (64). In all patients, the carcinoid tumor could be visualized by preoperative scintigraphy. By *ex vivo* autoradiography, silver grains were found at the plasma membrane, in the cytoplasmic areas among secretory granules and vesicular compartments, but also in the perinuclear area. This localization of $^{111}$In in close proximity to the cell nucleus is especially important for this short range Auger electron-emitting radioisotope to exert its cytotoxic effect

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in the form of DNA-double strand damage (see *Section II.B* and Ref. 64).

*1. Factors determining the uptake and cellular retention of radioactivity delivered via sst-mediated internalization.* [$^{111}$In-DTPA$^0$]octreotide is a sst$_2$-preferring ligand, which suggests an important role of sst$_2$ in determining the accumulation of radioactivity in tumor cells after internalization of the radioligand-receptor complex. Radiolabeled octapeptide SS-analogs are internalized in a high amount by sst-positive mouse and human tumor cells (63, 65–67). Evidence for a role of the sst$_2$ subtype in mediating the uptake of the radiopharmaceutical [$^{111}$In-DTPA$^0$]octreotide by sst-positive tumors is presented from studies showing that sst$_2$-expressing cells internalize SS (54), as well as octreotide (68). Moreover, sst$_2$ receptor expression correlates well with the relative uptake values of [$^{111}$In-DTPA$^0$]octreotide in patients with carcinoid tumor (69), as well as patients with neuroblastoma (70). On the other hand, on the basis of the high SS-internalization rates of the sst$_3$ and sst$_5$ subtypes, as reported by Hukovic *et al.* (54), it cannot be excluded that sst$_3$ and sst$_5$ might play a role as well. In fact, a role of the sst$_3$ subtype in the uptake of [$^{111}$In-DTPA$^0$]octreotide is evident from a recent study by our group demonstrating a significant uptake in a patient with a thymoma. *In vitro* studies revealed the absence of sst$_{2A}$, sst$_{2B}$, and sst$_5$ receptors and a predominant expression of sst$_3$ receptors in the tumor tissue (71). The hypothesis that sst subtypes other than sst$_2$ receptors may be involved in the uptake of [$^{111}$In-DTPA$^0$]octreotide *in vivo* is further underlined by the observation that sst scintigraphy visualized tumor sites in three patients with thyroid tumors (one MTC, one Hürthle cell adenoma, and one Hürthle cell carcinoma), which lacked sst$_2$ mRNA expression but expressed the other four sst subtypes (72), as well as in a patient with a pro-opiomelanocortin and CRH-expressing MTC (sst$_2$ negative; sst$_1$-, sst$_3$-, and sst$_5$-mRNA positive; Ref. 73).

As discussed in *Section I.A* (Table 1), octapeptide SS-analogs such as octreotide bind with high affinity to sst$_2$ and with lower affinity to sst$_3$ and sst$_5$ (74, 75). Therefore, both the affinity of the radioligand for the receptor and the efficiency of internalization of the radioligand-receptor complex can be important factors in determining the uptake of radioactivity in sst scintigraphy of sst-positive tumors. Moreover, the differential expression of sst subtypes in tumors (Table 2), as well as the level of sst subtype expression, may play a role. Until now, data on the differential internalization of SS by sst subtypes were derived from studies using cell lines transfected to overexpress the individual sst subtypes (*Section I.C*). Data on the internalization of SS ligands by (tumor) cells endogenously expressing sst subtypes are needed to evaluate the real significance of these findings for human sst-positive tumors. Because most human tumors express multiple sst subtypes, the development of novel sst subtype-selective agonists and antagonists will also be of help to unravel this question. Receptor-mediated endocytosis of SS-analogs is especially important when radiotherapy of human sst-positive tumors using radiolabeled SS-analogs is considered. Internalization of radioligand will result in a prolonged cellular retention of radioactivity, thus resulting in a prolonged exposure of the tumor cell to radiation. Human neuroendocrine tumor cells internalize the radioligand [$^{111}$In-DTPA$^0$]octreotide. However, this radiopharmaceutical may not be the most suitable compound to carry out radiotherapy because the Auger electrons emitter $^{111}$In has a low tissue penetration. In addition, a stable coupling of $\alpha$- or $\beta$-emitting isotopes to [DTPA$^0$]octreotide could not be achieved, which initiated the development of a novel compound, [DOTA$^0$,Tyr$^3$]octreotide, in which the diethylenetriamine pentaacetic acid (DTPA) molecule is replaced by another chelator, tetraazacyclododecane tetraacetic acid (DOTA), allowing a stable binding with the $\beta$-emitter yttrium-90 ($^{90}$Y) (76). Recently, we demonstrated that iodinated [DOTA$^0$,Tyr$^3$]octreotide is internalized in a high amount by mouse AtT20 pituitary tumor cells, as well as by human insulinoma cells (77). Internalization of iodinated [DOTA$^0$,Tyr$^3$]octreotide was approximately 5-fold higher, compared with the iodinated DTPA-coupled parent molecule, [DTPA$^0$,Tyr$^3$]octreotide. Therefore, coupling of the octreotide molecule to these chelating groups does not prevent internalization of the hybrid molecules. The high internalization rate of [DOTA$^0$,$^{125}$I-Tyr$^3$]octreotide *in vitro* was also evident from the very high uptake of this radioligand *in vivo* in sst-positive organs in rats. De Jong *et al.* (78) recently showed that the amount of [$^{90}$Y-DOTA$^0$,Tyr$^3$]octreotide internalized by sst-positive pancreatic tumor cells was significantly higher than that of [$^{111}$In-DOTA$^0$,Tyr$^3$]octreotide and [$^{111}$In-DTPA$^0$]octreotide (1.8- and 3.5-fold, respectively). Moreover, in eight patients with sst-positive tumors, a higher uptake value of [$^{111}$In-DOTA$^0$,Tyr$^3$]octreotide compared with that of [$^{111}$In-DTPA$^0$]octreotide was found in normal sst-positive organs like the spleen and pituitary gland, as well as in most tumors (79). If [$^{90}$Y-DOTA$^0$,Tyr$^3$]octreotide has the same characteristics of uptake in sst-positive cells, it may be a suitable radiopharmaceutical for sst-targeted radiotherapy.

After internalization of the receptor-radioligand complex, an important process is the retention of radioactivity within the tumor cells. For iodinated SS ligands, it seems clear that a significant proportion of radioactivity is rapidly excreted. This may be due in part to the excretion of radioligand degradation products, although recycling of the receptor-ligand complex may play a role as well. Recycling of SS receptors after being internalized has been demonstrated for sst$_2$ (80) and sst$_3$ (44, 81) receptors. Koenig *et al.* (80) also showed that biologically active SS agonists were excreted after being internalized by sst$_2$-expressing CHO cells. Therefore, trapping of radioisotopes into tumor cells may be an additional important mechanism determining the amount of uptake of radioligand that is used for sst scintigraphy and targeted radiotherapy. In this respect, Duncan *et al.* (82) previously demonstrated that [$^{111}$In-DTPA$^0$]octreotide is delivered *in vivo* to pancreatic tumor cell lysosomes and proposed that lysosomes play a critical role in the cellular physiology of radiolabeled SS-analogs. The internalized [$^{111}$In-DTPA$^0$]octreotide was shown to be metabolized to $^{111}$In-DTPA-D-Phe *in vivo* (83, 84). Accumulation of radioactivity in nuclear-lysosomal density gradient fractions was also found in neuroblastoma cells exposed *in vitro* to the radioligand (85). For the radioligand [$^{90}$Y-DOTA$^0$,Tyr$^3$]octreotide, it remains to be determined whether similar

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processes occur. Finally, uptake of radiolabeled [DTPA$^0$]-octreotide in rats and humans (61, 86), as well as that of [DOTA$^0$,Tyr$^3$]octreotide in rats (87), demonstrated a bell-shaped curve, dependent upon the amount of injected peptide. Studies to determine the optimal peptide mass for uptake of radioactivity in human tumors after the injection of radiolabeled SS-analogs are ongoing (87).

As shown in Table 1, different octapeptide SS-analogs such as octreotide, lanreotide, and vapreotide (RC-160) interact with the same subclass of sst subtypes ($sst_2$, $sst_3$, and $sst_5$). Nevertheless, slightly different affinities for the different sst subtypes have been found (Table 1). However, *in vivo* studies in rats (88) and humans (89) comparing uptake values of [$^{111}$In-DTPA$^0$]RC-160 and [$^{111}$In-DTPA$^0$]octreotide showed that [$^{111}$In-DTPA$^0$]RC-160 has no additional value for scintigraphy. In fact, the use of [$^{111}$In-DTPA$^0$]RC-160 is associated with higher background activity (89). Another recently developed radiopharmaceutical, $^{111}$In- or $^{90}$Y-labeled DOTA-lanreotide, bound with high affinity to $hsst_2$–$hsst_5$ and with low affinity to $hsst_1$ expressed in COS-7 cells, suggesting that this radiolabeled peptide may also be useful for sst scintigraphy or radiotherapy (90). Apart from differences in the affinity profiles of unlabeled SS-analogs due to structural differences, radiolabeling of such analogs has major effects on binding affinity for the different human sst subtypes as well (91). Yttrium labeling of [DOTA-Tyr$^3$]octreotide, DOTA-lanreotide, and DOTA-RC-160 significantly increases binding affinities for $sst_3$ and $sst_5$ receptors. Such differences, in combination with the high internalization rates of $sst_3$ and $sst_5$ receptors (54), could very well account for the higher cellular uptake values *in vivo* and *in vitro* of [$^{90}$Y-DOTA$^0$,Tyr$^3$]octreotide, compared with [$^{111}$In-DTPA$^0$,Tyr$^3$]octreotide and [$^{111}$In-DTPA$^0$]octreotide (77–79). Therefore, several characteristics of SS-analogs developed for sst scintigraphy and radiotherapy, such as small structural modifications, chelator substitution, or type of radioisotope, considerably affect binding affinity (91).

Preclinical studies have shown that down-regulation of SS receptors may occur during agonist exposure. On the other hand, agonist-induced up-regulation of sst expression has been demonstrated as well (see *Section III.D.1*). Agonist-induced regulation of tumoral sst expression may theoretically influence the results of sst scintigraphy and the efficacy of targeted radiotherapy. The few available clinical data on this issue add to the equivocal data regarding up-regulation and/or down-regulation of SS receptors upon exposure to SS or SS-analog treatment. In five patients with metastatic MTC who were studied before and after 3 months of therapy with a high dose of octreotide, tumor/background ratios determined by sst scintigraphy were reduced in 14 of 18 metastases, suggestive of a down-regulation of SS receptors by octreotide therapy (92). Moreover, reduced orbital uptake of octreoscan was observed in 10 patients with thyroid eye disease after 3 months of treatment with lanreotide or octreotide (93). On the other hand, in patients with a somatostatinoma, the tumors can be visualized by sst scintigraphy (94, 95), suggesting that a complete sst down-regulation does not occur in this type of tumor. Finally, Dorr *et al.* (96) reported decreased uptake of octreoscan in the liver, spleen, and kidney during continuous octreotide therapy, whereas tumor uptake values were increased simultaneously in five patients with metastatic carcinoid disease and decreased in one patient with advanced MTC. In conclusion, homologous down-regulation of sst expression may be (tumor) cell type specific, as was already evident from experimental studies (see *Section III.D.1*).

In conclusion, it is well established now that radiolabeled SS-analogs, including those that are used for sst scintigraphy and sst-targeted radiotherapy, can be internalized by sst-positive tumor cells. Several mechanisms may determine the amount of uptake of radiolabeled SS-analogs. These include stability of the radioligand, the expression levels of individual sst subtypes, the affinity of the radioligand for the sst (subtype), the efficiency of receptor internalization and recycling that may be different between sst subtypes, the final trapping of the radioisotopes within the tumor cells, as well as the mass of the injected peptide (summarized in Table 3).

### *B. SS receptor-targeted radiotherapy*

*1. Preclinical evidence.* Several preclinical studies have demonstrated tumor growth-inhibitory effects after treatment with radiolabeled SS-analogs. In athymic mice bearing sst-positive PC-3 prostatic adenocarcinoma, Zamora *et al.* (97) showed that intratumoral injections with seven 200-$\mu$Ci doses of the $\beta$-emitting $^{188}$Re-RC-160 SS-analog reduced tumor size by 90%. Additionally, a significantly higher proportion of survivors was observed in the group treated with $^{188}$Re-RC-160. In this study, treatments were initiated 19 d after inoculation of PC-3 tumor cells when the animals carried solid tumors (500–1000 mm$^3$). In addition, three serial treatments with regionally injected 200 $\mu$Ci $^{188}$Re-RC-160 decreased tumor burdens in experimental models of H-69 human small cell lung cancer cells or ZR-75-1 mammary adenocarcinoma cells xenografted into the pleural cavity of athymic mice (98). Tumor ablation was observed in up to 60% of the animals bearing H69 tumors and in 40% of the animals bearing ZR-75-1 tumors (98). In another study in nude mice bearing solid sst-positive AR42J pancreatic tumors, a single treatment with 500 $\mu$Ci of another SS-analog, radiolabeled with the $\beta$-emitting isotope $^{90}$Y, *e.g.*, [$^{90}$Y]SDZ413, decreased tumor mass by 75% 8 d after injection and prolonged survival, although tumor regrowth was observed after 2 wk of treatment (99). More recently, a significant radiotherapeutic effect of the $^{90}$Y-labeled SS-analog [$^{90}$Y-DOTA-D-Phe$^1$,Tyr$^3$]octreotide was demonstrated by the same group of investigators in rats bearing solid sst-positive pancreatic CA 20948 tumors (76). A single iv administration of 10 mCi/kg [$^{90}$Y-DOTA-D-Phe$^1$,Tyr$^3$]octreotide resulted in a complete remission of

TABLE 3. Factors important in determining the amount of tumoral uptake of radiolabeled SS-analogs

1. Stability of the radioligand
2. Density of SS receptors expressed on tumors
3. Type of SS receptors expressed by tumors
4. Affinity of radioligand for sst subtype(s) expressed by tumors
5. Efficiency of receptor subtype-mediated internalization of radioligand
6. Trapping of radioisotopes within the tumor cell
7. Mass of the injected radiopharmaceutical

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the tumors in five of seven (71%) tumor-bearing rats. In this study, no tumor regrowth had occurred even 8 months post injection. This pancreatic CA 20948 tumor expressed a high level of $sst_2$ mRNA and a low level of $sst_5$ mRNA, suggesting that the radiotherapeutic effect of [$^{90}$Y-DOTA-D-Phe$^1$,Tyr$^3$]octreotide was mediated via targeting the $sst_2$ receptor subtype. [$^{90}$Y-DOTA-D-Phe$^1$,Tyr$^3$]octreotide is the radiopharmaceutical that is currently being tested in ongoing clinical phase I and II trials (see *Section II.B.2*).

Whereas solid experimental tumor models were used in the above studies, Slooter *et al.* (100) recently studied the radiotherapeutic effect of [$^{111}$In-DTPA$^0$]octreotide in a rat model of hepatic metastasis of different tumor cell lines. In this study, the development of hepatic metastases was determined 21 d after injection of sst-positive or sst-negative tumor cells into the vena porta in rats. Tumor-bearing rats were treated twice (d 1 and d 8) with 370 MBq of [$^{111}$In-DTPA$^0$]octreotide. These investigators demonstrated a significant reduction in the number of liver metastases by this treatment regimen in the sst-positive tumor model, but not in the sst-negative tumors. This suggests that the presence of sst on the tumor cells is required for effectiveness of treatment with radiolabeled SS-analogs. This was further confirmed by their observation that the radiotherapeutic effect of [$^{111}$In-DTPA$^0$]octreotide could be blocked by coinjection with a sst-saturating dose of unlabeled octreotide (100). As indicated above, it should be mentioned that $^{111}$In is not a $\beta$-emitting radioisotope, but emits $\gamma$-rays, internal conversion, and Auger electrons. Internal conversion and Auger electrons have a medium- to short-range tissue penetration (200–500 $\mu$m and 0.02–10 $\mu$m, respectively), and it is suggested that these radiochemical properties of $^{111}$In cause the radiotherapeutic effect of [$^{111}$In-DTPA$^0$]octreotide. Because in this study the tumor load was relatively small, studies of the radiotherapeutic effect of [$^{111}$In-DTPA$^0$]octreotide in experimental models of more advanced stages of tumor development are required (100).

Recently, a novel $^{177}$lutetium ($^{177}$Lu, a low-energy $\beta$-particle and $\gamma$-emitter) radiolabeled SS-analog, *i.e.*, [$^{177}$Lu-DOTA,Tyr$^3$]octreotate, has been proven to be a very promising radiopharmaceutical. Preclinical studies in rats bearing CA 20948 pancreatic tumors demonstrated even a 100% cure of small ($\leq$1 cm$^2$) tumors after two repeated doses of 277.5 MBq or one single dose of 555 MBq of [$^{177}$Lu-DOTA,Tyr$^3$]octreotate (101). In rats with larger tumors ($\geq$1 cm$^2$; range, 1.4–10 cm$^2$), cure rates between 40% and 50% were observed (102). In rats bearing AR42J pancreatic tumors, which had a more favorable uptake compared with the CA 20948 model, treatment with 555 MBq of [$^{177}$Lu-DOTA,Tyr$^3$]octreotate resulted in an almost 100% cure, irrespective of the tumor size (102). On the basis of the distinct properties of the two radionuclides and the results of preclinical studies, this group of investigators proposed the use of a combination of $^{90}$Y- and $^{177}$Lu-labeled SS-analogs, because $^{90}$Y is particularly effective in large tumors and $^{177}$Lu seems most effective in small tumors (102). Preliminary results in a rat model with tumors of more than one size indeed showed longer survival rates with the combined treatment, compared with treatment with the $^{90}$Y- or $^{177}$Lu-labeled SS-analogs alone (103).

It is well known that tumor cells display various degrees of sensitivity to radiation. Adenovirus-based transfer of wild-type (wt) p53 tumor suppressor gene sensitizes ovarian tumor cells to radiation-induced apoptosis (104). In addition, overexpression of the tumor suppressor gene Bax can sensitize tumor cells to radiation, as well as to chemotherapy-induced apoptosis (105, 106). Of particular importance in this respect are recent studies demonstrating that octreotide induces wt p53 and Bax in MCF-7 human breast cancer cells (107). SS-mediated induction of wt p53 and apoptosis is selectively induced via $sst_3$ (108), in contrast to p53-independent, retinoblastoma protein-mediated signaling of cell cycle arrest (109, 110). The majority of human sst-positive tumors express $sst_3$ (*Section I*). It cannot be excluded, therefore, that the therapeutic potential of internalized radionuclide may be limited by the lack of expression of a functional p53 or $sst_3$. On the basis of their observations, Sharma and Srikant (107) suggested that $\alpha$- or $\beta$-emitting octreotide-tagged radionuclides should elicit maximal cytotoxic response due not only to radiation-induced damage after internalization, but also to the triggering of apoptosis via the induction of wt p53 and Bax by receptor-mediated signaling. Moreover, on the basis of these data, it is predicted that treatment with SS-analogs alone or in combination with radiation and/or chemotherapy will be most effective in treating wt p53- and sst-expressing tumors not only of the breast but also of other organs (107).

Taking the preclinical studies together, it can be concluded that radiotherapy using radiolabeled SS-analogs is effective in experimental sst-positive tumor models and that sst-targeted radionuclide therapy may be a feasible approach to treat patients with advanced, metastatic sst-positive neuroendocrine tumors.

*2. Clinical evidence.* In 1989, the technique of sst scintigraphy to visualize sst-positive tumors in man was developed using the radiolabeled SS-analog [$^{123}$I-Tyr$^3$]octreotide (12). Because the use of this radiopharmaceutical had a number of drawbacks (*i.e.*, expensive, lack of availability, short physical half-life, and predominant hepatic clearance resulting in accumulation of radioactivity in liver, gall bladder, bile ducts, and gastrointestinal tract; Ref. 2), novel SS-analogs were developed to circumvent these disadvantages. As described above, the most widely used SS-analog for sst scintigraphy is a DTPA-coupled octreotide. The radioisotope $^{111}$In binds with very high affinity to the DTPA molecule, and [$^{111}$In-DTPA$^0$]octreotide has proved to be a highly suitable radiopharmaceutical for the detection of sst-positive tumors by $\gamma$-camera scintigraphy (13). Apart from its use in sst scintigraphy, [$^{111}$In-DTPA$^0$]octreotide has been used for radiotherapeutic application as well (111, 112). Although no controlled trials have been performed with [$^{111}$In-DTPA$^0$]octreotide, preliminary reports demonstrate a certain degree of efficacy of this radiopharmaceutical in the treatment of selected, high sst-expressing metastasized neuroendocrine tumors. Krenning *et al.* (111) reported treatment with [$^{111}$In-DTPA$^0$]octreotide, up to maximal cumulative patient doses of 74 GBq in a phase I trial of 30 end-stage patients with neuroendocrine tumors that all demonstrated recent progression. They reported promising beneficial effects on clinical symptoms, hormone pro-

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duction, and tumor size. In 21 patients who received a cumulative dose of more than 20 GBq, 8 patients showed stabilization of disease, and 6 others demonstrated a reduction in tumor size. In a few patients, a transient decline in platelet counts and lymphocyte subsets occurred (111). More recently, this group of investigators reported on 40 evaluable patients treated with doses of at least 20 GBq up to 160 GBq. In 21 patients, therapeutic effects were observed: partial remission in 1, minor remissions in 6, and stabilization of previously progressive disease in 14 patients. On the basis of the observation that three of six patients, who received more than 100 GBq of [$^{111}$In-DTPA$^0$]octreotide, developed myelodysplastic syndrome or leukemia, 100 GBq was considered to be the maximal tolerable dose (113). In another study, 14 patients with sst-positive malignancies of different types were treated with two monthly doses of 180-mCi iv injections of [$^{111}$In-DTPA$^0$]octreotide. Clinical benefit occurred in 6 of 10 patients with gastroenteropancreatic tumors, objective partial radiographic responses occurred in 2 of 14 patients, and significant tumor necrosis occurred in 6 of 10 patients. Possible treatment-related toxicity included myelosuppression (114). In a patient with a midgut carcinoid tumor, treatment with therapeutic doses of [$^{111}$In-DTPA$^0$]octreotide induced symptomatic relief, including a slight reduction in levels of the main tumor marker, urinary 5-hydroxyindolacetic acid (5-HIAA). Again, a slight reduction in leukocyte counts was observed as adverse reaction (112). These data suggest that radiotherapy with high doses of [$^{111}$In-DTPA$^0$]octreotide might be useful as a therapeutic agent in patients with sst-positive malignancies. The therapeutic effect of [$^{111}$In-DTPA$^0$]octreotide seems due to the emission of Auger and conversion electrons having a low tissue penetration (see *Section II.B.1* and Ref. 111). $^{111}$In may therefore not be the most optimal radionuclide for sst-targeted radionuclide therapy. For this, novel DOTA-chelated SS-analogs have been synthesized, which allow a fixed binding of β-emitting radionuclides, such as $^{90}$Y. Recently, clinical trials using [$^{90}$Y-DOTA$^0$,Tyr$^3$]octreotide as well as another DOTA-coupled SS-analog, *e.g.*, [$^{90}$Y-DOTA]lanreotide, have been initiated. Preliminary results with [$^{90}$Y-DOTA$^0$,Tyr$^3$]octreotide demonstrated promising effects in patients with different types of sst-positive neuroendocrine tumors (115). Multiple treatments with [$^{90}$Y-DOTA$^0$,Tyr$^3$]octreotide resulted in stable disease in three of six patients and partial remission in the remaining patients. In two of four patients who received a single treatment with [$^{90}$Y-DOTA$^0$,Tyr$^3$]octreotide, tumor glucose uptake was reduced, whereas the other two showed clinical improvement and stable disease. In a larger group of patients with advanced sst-positive tumors of different origin, a [$^{90}$Y-DOTA$^0$,Tyr$^3$]octreotide intrapatient dose escalation study has been performed. In this study, 29 patients received 4 or more single doses of [$^{90}$Y-DOTA$^0$,Tyr$^3$]octreotide with ascending activity at intervals of approximately 6 wk. Preliminary results showed disease stabilization in 20 of the 29 patients, partial remission in 2, a reduction in tumor mass of less than 50% in 4, and progression of tumor growth in 3 patients (116). However, a significant proportion of the patients (5 of 29, or 17%) developed renal and/or hematological toxicity. Studies directed to reduce renal toxicity, *i.e.*, amino acid infusions, are ongoing (116). Paganelli *et al.* (117, 118) reported favorable preliminary results regarding tumor growth using [$^{90}$Y-DOTA$^0$,Tyr$^3$]octreotide as well. A recent phase II study, including 41 patients with neuroendocrine, gastroenteropancreatic, and bronchial tumors and 82% of patients with therapy-resistant and progressive disease, showed an overall response rate of 24%. Side effects included grade III (NCIGC) pancytopenia in 5% and vomiting shortly after injection in 23%. No grade III-IV renal toxicity was observed (119). To evaluate the clinical benefit and objective response rate of high-dose [$^{90}$Y-DOTA$^0$,Tyr$^3$]octreotide treatment (4 equal iv injections totaling 7.4 GBq/m$^2$ with renal protection), a phase II study in 39 patients with progressive neuroendocrine, gastropancreatic, and bronchial tumors was performed (120). The results showed an overall objective response rate of 23% (World Health Organization criteria: complete remission in 5%, partial remission in 18%, stable disease in 69%, progressive disease in 8%). In the patients with endocrine pancreatic tumors, objective response rate was 38% (13 patients). The overall clinical benefit in this study was 63%. Side effects were grade III or IV lymphocytopenia (23%), grade III anemia (3%), and grade II renal insufficiency (3%). Finally, in a patient with metastatic gastrinoma treated with [$^{90}$Y-DOTA]lanreotide, a 25% reduction in liver metastases as indicated by computed tomography was observed (121). In conclusion, the results of sst-targeted radiotherapy with [$^{90}$Y-DOTA$^0$,Tyr$^3$]octreotide are most encouraging and extend the therapeutic options in the treatment of patients with sst-positive neuroendocrine tumors.

Very recently, Kwekkeboom *et al.* (122) introduced a novel DOTA-tagged SS-analog, *e.g.*, [DOTA$^0$,Tyr$^3$]octreotate (in which the C-terminal threoninol is replaced with threonine), radiolabeled with the β- and γ-emitting $^{177}$Lu, as a potential promising radiotherapeutical with a 3- to 4-fold higher tumoral uptake of radioactivity compared with [$^{111}$In-DTPA$^0$]octreotide. [DOTA$^0$,Tyr$^3$]octreotate has a 9-fold increased affinity for sst$_2$, compared with [DOTA$^0$,Tyr$^3$]octreotide, and a 6- to 7-fold increase in affinity for their yttrium-loaded counterparts (91). Preliminary promising results using this radiopharmaceutical have been reported (102, 103).

### *C. SS receptor-targeted chemotherapy: preclinical evidence*

The wide spectrum of adverse reactions when treating patients with advanced, metastatic tumors with chemotherapeutic agents are caused by the severe toxicity of these agents to normal cells. Like peptide receptor-targeted radiotherapy, targeted chemotherapy to deliver the chemotherapeutic compounds selectively to tumor cells might be a promising approach as well. Schally and Nagy (123) pioneered this concept with the development of cytotoxic analogs of LHRH, cytotoxic bombesin analogs, and cytotoxic SS-analogs, to treat LHRH receptor-, bombesin receptor-, and sst-positive tumors, respectively. This group of investigators provided preclinical evidence for the effectiveness of cytotoxic LHRH analogs in experimental models of human ovarian, mammary, or prostatic cancer (123), as well as for the effectiveness of cytotoxic bombesin analogs in the treatment of experimental models of bombesin receptor-positive small cell lung carcinoma, colorectal, gastric, pancreatic, mammary, and prostatic cancers (123). Schally and co-work-

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ers (124, 125) synthesized two different cytotoxic SS-analogs, code-named AN-51, consisting of methotrexate linked to the N terminal of the octapeptide SS-analog RC-121 and AN-238, which is the RC-121 analog linked to a highly potent derivative of doxorubicin, *e.g.*, 2-pyrrolinodoxorubicin. Both the AN-51 and AN-238 compounds had intermediate binding affinities to sst-positive tissues *in vitro*, in comparison with the RC-121 compound alone, suggesting that coupling of the chemotherapeutic compound to the SS-analog slightly reduced their binding properties (124, 125). The binding affinity of the AN-238 compound for rat pituitary membrane SS receptors was 23.8 nM, which is comparable to the binding affinities of several DTPA- and DOTA-coupled SS-analogs to $hsst_2$ receptors (91). In preclinical studies, it was demonstrated that both the AN-51 and AN-238 compounds inhibited tumor growth in experimental tumor models. In nude mice transplanted with the human Mia PaCa-2 pancreatic tumor, AN-51 significantly inhibited tumor growth, whereas the chemotherapeutic compound alone, methotrexate, or RC-121 alone had no significant inhibitory effect (124), and with methotrexate alone displaying a much higher toxicity compared with AN-51. Thereafter, this group tested the cytotoxic properties of the AN-238 compound, which displayed a very high toxicity in sst-positive cells *in vitro*. Potent tumor growth inhibitory properties of AN-238 were observed in many experimental mouse and rat models of human breast cancer, prostate cancer, ovarian cancer, small cell lung cancer, pancreatic cancer, renal cell cancer, as well as glioblastoma (126–133). Again, a much higher toxicity and lower or absent effectiveness on tumor growth was observed in animals treated with the cytotoxic radical alone. In these animal studies, the major side effect of treatment with cytotoxic SS-analogs was a transient fall in white blood cell counts. In conclusion, sst-targeted chemotherapy is effective in preclinical tumor models and seems a highly promising approach as well to treat sst-positive tumors. The sst-targeted chemotherapy may result in a chemotherapeutic approach using lower dosages of the chemotherapeutic compound and thus lower toxicity. Until now, however, no clinical trials have been reported using targeted LHRH, bombesin, or SS-analogs. In addition, evidence will have to be provided that cytotoxic SS-analogs can also be internalized by sst-positive tumor cells. As for the concept of sst-targeted radiotherapy, the efficacy of sst-targeted chemotherapy will be determined by the amount of uptake of the cytotoxic radicals by the tumors. Moreover, the effect of cytotoxic SS-analog treatment on the function of normal sst-expressing cells is to be determined.

## III. Tachyphylaxis and Resistance to SS

### *A. Introduction*

Concomitant with the widespread distribution of sst throughout central and peripheral tissues, the acute administration of SS or its analogs induces a large number of mainly inhibitory effects (8, 9). Nevertheless, these initially potent responses diminish with continued exposure (9, 11). The different mechanisms that are potentially involved in this adaptation or tachyphylaxis to continuous exposure to SS or SS-analogs may be associated with processes such as receptor phosphorylation, G protein uncoupling, receptor internalization, and degradation. This has been reviewed extensively (8, 9). Different from these physiological responses to continued SS exposure is the response of neuroendocrine tumor cells. Patients with certain types of sst-positive tumors (*e.g.*, GH-secreting pituitary adenomas, islet cell tumors, and carcinoids) can be treated for many months to years with the current clinically available SS-analogs. The long-term control of hormonal hypersecretion and/or tumor growth by treatment with SS-analogs may vary considerably, however, among patients. *Section III* focuses particularly on tachyphylaxis and resistance to treatment with the different available formulations of SS-analogs, as well as the potential mechanisms involved herein. Although the direct fundamental evidence for the clinical observations of tachyphylaxis is relatively weak, several potential mechanisms determining cellular responsiveness to SS are discussed.

### *B. Tachyphylaxis of pathological hormone secretion*

*1. Pituitary adenomas.* Whereas normal hormone secretion shows tachyphylaxis after continuous receptor activation within hours to days (9), pathological hormone secretion by sst-positive tumor cells can be inhibited during significantly prolonged periods. In about half of the patients with GH-secreting pituitary adenomas, serum GH and IGF-I levels are normalized by octreotide treatment (134). Escape from SS-analog therapy has not been observed in this type of patient, even after many years of continuous treatment (135). Figure 2A shows a typical example of the persistent suppression of serum IGF-I levels in an acromegalic patient during a period of 8 yr of treatment with three times daily injections of 50–100 μg octreotide. The persistently lowered IGF-I levels seem not to be caused by radiotherapeutic effects because drug withdrawal after 5 yr of treatment resulted in an instant rise in IGF-I levels and immediate recurrence of signs and symptoms. Moreover, although no desensitization to continuous sc treatment with octreotide is observed, acromegalic patients treated with long-acting formulations of SS-analogs, *i.e.*, long-acting repeatable octreotide administration (Refs. 135a–135c) or slow-release lanreotide (136) did not show any signs of tachyphylaxis to treatment periods up to 3 yr as well. To our knowledge, only one rare case of acromegaly showing desensitization to octreotide has been described so far (137). Partial tachyphylaxis to SS-analogs was reported in a patient with acromegaly. In this patient, previously treated with $^{90}Y$ implant, external radiotherapy, and three daily sc injections with octreotide, GH levels progressively rose after switching to lanreotide and depot octreotide (Sandostatin LAR, Novartis Pharmaceuticals Corp., Basel, Switzerland). Interestingly, there were no signs of tumor growth or alterations in sst status as determined by [$^{111}In$-$DTPA^0$]octreotide scintigraphy (138). Octreotide withdrawal for 24 h in this patient resulted in a 64% increased sensitivity in terms of inhibition of GH levels by recommencing octreotide treatment, suggesting that changes in receptor function or on the receptor signal transduction cascade play a role, rather than changes in receptor expression (138).

The majority of TSH-secreting and clinically nonfunction-

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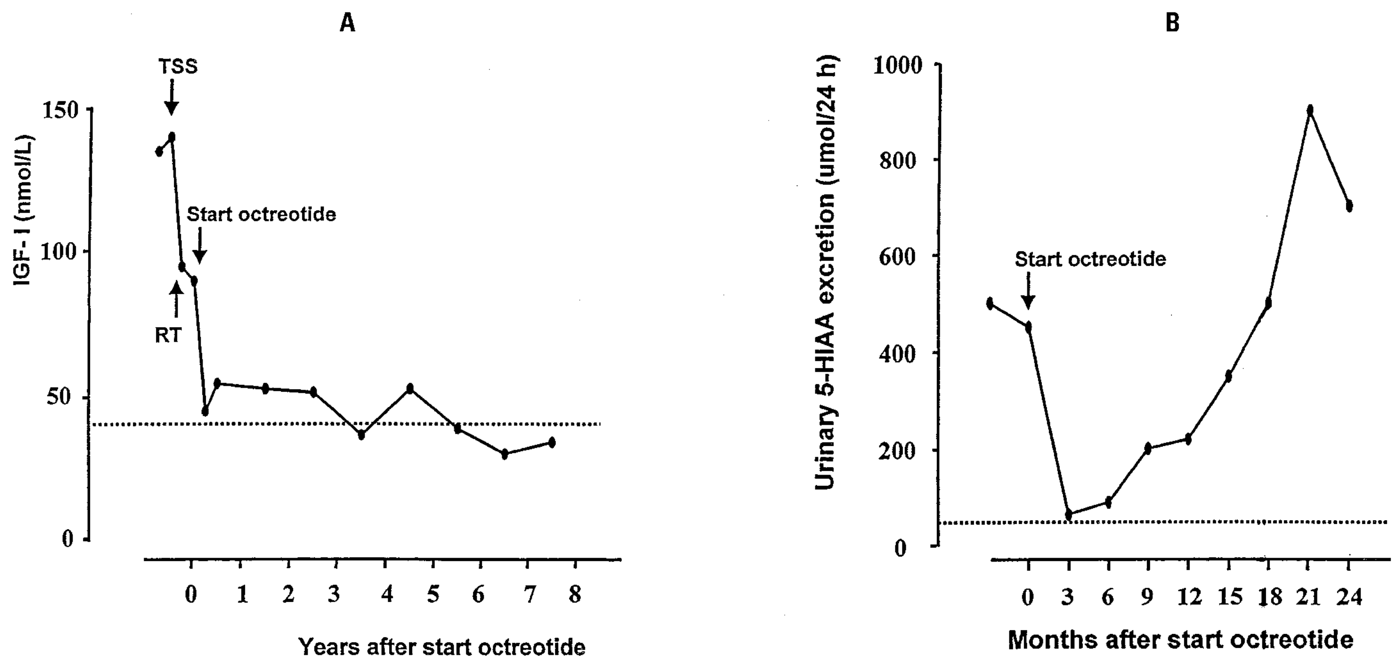


FIG. 2. Absence of tachyphylaxis to octreotide therapy in a patient with a GH-secreting pituitary adenoma (A) and desensitization in a patient with metastatic carcinoid tumor (B). A, Effect of octreotide therapy on serum IGF-I level in a patient with a GH-secreting pituitary adenoma. A 77-yr-old man transsphenoidally operated (TSS) for a GH-secreting macroadenoma that had resulted in active acromegaly. Two months after incomplete surgery of the tumor, external radiotherapy (RT) was applied. Successively, octreotide therapy was started 6 months after surgery at a dose of 100 $\mu$g three times daily. This therapy had resulted in a prolonged suppression of serum IGF-I levels (N $<$ 43 nmol/liter) and disappearance of signs and symptoms of active acromegaly. Octreotide therapy has been continued for more than 8 yr. The dose could be reduced to 50 $\mu$g three times daily. Discontinuation of therapy resulted in an increase of serum IGF-I levels and immediate recurrence of signs and symptoms of active acromegaly. *Dotted line* shows the upper normal limit of serum IGF-I levels. B, Effect of octreotide therapy on urinary 5-HIAA levels in a patient with a metastatic carcinoid tumor. A 66-yr-old man, operated for a metastatic carcinoid tumor of the small intestine with abdominal lymph node metastases, hepatic metastases, and the malignant carcinoid syndrome. Urinary 5-HIAA levels were greatly elevated (N $<$ 40 $\mu$mol/24 h). Therapy with octreotide was started at a dose of 100 $\mu$g three times daily. This therapy initially resulted in a reduction of attacks of flushing and improvement of diarrhea, which was accompanied by more than 50% reduction (but not normalization) of urinary 5-HIAA levels. However, after 4–6 months of therapy, the patient developed resistance to therapy: the flushing attacks, frequency of diarrhea, and urinary 5-HIAA levels gradually increased despite increasing the dose to 500 $\mu$g three times daily. In addition, a slight increase of tumor mass was observed. The *dotted line* represents the upper normal limit of urinary 5-HIAA levels.

ing pituitary adenomas also express sst (139, 140). Octreotide treatment of patients with TSH-secreting pituitary adenomas results in a lowering of TSH levels and normalization of $T_4$ levels in 73% of the patients. In contrast to patients with GH-secreting pituitary adenomas, in this series of 52 patients an escape from therapy was observed in 5 patients (10%). This loss of sensitivity to the inhibitory effect of octreotide on TSH levels was observed in two patients receiving short-term therapy and in three patients receiving long-term therapy (139). Overall, Beck-Peccoz *et al.* (141) reported tachyphylaxis in 22% of the patients with a response to increasing octreotide doses, whereas subsequent escape from the inhibitory effects was observed in 10% of the cases. The role of SS-analogs in the treatment of patients with clinically nonfunctioning pituitary adenomas is less well established, whereas octreotide seems not of benefit in the treatment of patients with ACTH-secreting pituitary adenomas or prolactinomas (135). This may be due to either the absence of sst on the tumor cells or the absence of expression of particular sst subtypes (135).

*2. Islet cell tumors and carcinoids.* In striking contrast to the absence of the occurrence of tachyphylaxis of inhibition of hormone secretion by octreotide in patients with GH-secreting pituitary adenomas are the observations in patients with islet-cell tumors and carcinoids. In the majority of patients with metastatic carcinoids, VIPomas, gastrinomas, insulinomas, and glucagonomas, treatment with octreotide induces a rapid improvement of clinical symptomatology, such as diarrhea, dehydration, flushing attacks, hypokalemia, peptic ulceration, hypoglycemic attacks, and necrotic skin lesions (142–145). On the other hand, the majority of the patients show desensitization of the inhibition of the secretion of tumor-related hormones by octreotide within weeks to months. This effect may be initially reversed by increasing the dosage of octreotide, but eventually the drug becomes ineffective in all patients (11). In a series of 57 patients with the carcinoid syndrome, 23 patients escaped from octreotide therapy after periods ranging from 1 wk to 12.5 months (median, 4 months), whereas the other responding patients could be controlled for periods extending to 2.5 yr. The estimated mean duration of response to octreotide therapy in the whole group of responding patients was approximately 1 yr (146). Figure 2B shows a typical example of tachyphylaxis of the inhibitory effect of octreotide (100 $\mu$g three times per day) on urinary 5-HIAA levels in a patient with a metastatic carcinoid tumor. An escape from octreotide treatment was seen after 3 months of therapy. Increasing the dose of

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octreotide (to 500 μg three times per day) was not beneficial in this particular patient. The potential mechanisms responsible for this desensitization, as well as for the considerable variability in the duration of the responses to octreotide therapy, are not known at present. The relatively long timeframe of this escape suggests mechanisms other than G protein uncoupling or internalization are involved. It has been suggested that this loss of sensitivity of endocrine cancers to octreotide is possibly associated with the outgrowth of clones of tumor cells that lack sst rather than with a transient down-regulation of these receptors (147). Moreover, it is not known why pituitary GH-secreting pituitary adenomas do not show tachyphylaxis to octreotide or lanreotide treatment, whereas the majority of patients with metastatic carcinoids, VIPomas, gastrinomas, insulinomas, and glucagonomas eventually desensitize. Possibly, SS-analog treatment of patients with GH-secreting pituitary adenomas induces an up-regulation of sst in the GH-secreting tumor cells, whereas other types of tumors display down-regulation of sst upon prolonged agonist exposure. As will be discussed in *Section III.D.1*, up-regulation and/or down-regulation of sst expression may be sst subtype dependent. As has been discussed in *Section I*, the majority of human sst-positive tumors express multiple sst subtypes, often with overlapping patterns. Therefore, potential tissue-specific desensitization and/or down-regulation of sst subtypes or, alternatively, tissue-specific up-regulation of the octreotide-responsive sst subtypes 2, 3, and 5 induced by prolonged agonist treatment may account for continued responsiveness of GH-secreting pituitary adenomas to SS agonists.

### *C. Escape from antiproliferative effects*

Apart from regulating neurotransmission and secretion, SS and its analogs may inhibit cell proliferation in normal and tumoral tissues as well. Evidence for inhibition of tumor cell proliferation by SS-analogs is based primarily on studies using experimental sst-positive tumor models (2, 3). However, in a number of these studies, tumor growth is only delayed, because after a certain treatment period the tumors start to grow more rapidly, resulting in growth curves that parallel tumor growth in untreated control animals, indicating escape from SS-analog therapy (148–150). In the model of the transplantable prolactin (PRL)-secreting pituitary tumor (149), we observed during the first 2 wk of treatment with the SS-analog octreotide a significant reduction in tumor growth. After 2 wk of treatment, however, tumor growth rates in untreated and octreotide-treated animals were parallel and not significantly different. One of the mechanisms underlying this tachyphylaxis may be a down-regulation of SS receptors on the tumor cells. In primary cultures of PRL-secreting 7315b cells, an incubation with octreotide for 1 wk inhibited both the growth and hormone secretion in a parallel and dose-dependent fashion. However, prolonged (5 wk) continuous exposure to octreotide (0.1 nM to 1 μM) resulted in tachyphylaxis with respect to the inhibition of PRL secretion. In a stable cell line derived from this 7315b tumor, long-term exposure to octreotide induced a loss of sensitivity with respect to both PRL secretion and cell growth. This loss of sensitivity was accompanied by a complete down-regulation of SS binding sites on the tumor cells. In this 7315b sst-expressing tumor model, clonal selection of sst-negative cells was not the cause of desensitization, because withdrawal of treatment from desensitized cells resulted in a reappearance of sst and the sensitivity to octreotide (151). A significant reduction in sst numbers induced by octreotide treatment has also been demonstrated in Syrian hamsters bearing transplanted insulinomas. Twice-daily injections with octreotide for 3 d resulted in a dose-dependent reduction in sst numbers on the insulinomas (2). On the other hand, the occurrence of tachyphylaxis to treatment with SS-analogs can be tumor cell type specific. *In vivo* studies by other groups showed an increase in SS-binding on experimental tumors treated with SS-analogs. Treatment of human MKN45 gastric carcinoma xenografts in nude mice for 5 wk with the SS-analog RC-160 significantly inhibited tumor growth without the occurrence of an escape. Daily sc injections with RC-160 even induced a significant up-regulation of sst in these tumors after 4–5 wk, which in this particular tumor model may be beneficial in maintaining the inhibitory effects on tumor growth (152). A comparable up-regulation of sst binding sites has been demonstrated in AR4-2J pancreatic tumor-bearing mice, in which continuous treatment (7 d) with a low dose of octreotide, administered via octreotide-containing osmotic minipumps, induced an increase in the number of tumoral sst binding sites (153). In contrast to this up-regulation of sst binding sites on pancreatic AR4-2J tumors by continuous *in vivo* treatment with low doses of octreotide, discontinuous (twice daily) sc injections of octreotide resulted in a down-regulation of sst expression (153). After removal of octreotide *in vitro*, a total recovery of [$^{125}$I-Tyr$^{3}$]octreotide binding was observed within 24 h. This recovery was dependent on protein synthesis, making *de novo* receptor synthesis necessary for the recovery process (153). RT-PCR analysis revealed that AR4-2J cells expressed $sst_2$ receptor mRNA only. In fact, these authors concluded that continuous treatment with a low dose of octreotide might improve the efficacy of long-term octreotide therapy. These data suggest that in a single tumor model the experimental conditions may determine whether $sst_2$ receptors are either down-regulated or up-regulated. In conclusion, the escape from the tumor growth-inhibitory effects of SS-analogs suggests that prolonged exposure to agonists may be due to sst down-regulation. Moreover, in some tumor models an up-regulation of sst expression after agonist exposure has been observed, which might explain prolonged responsiveness to SS-analogs. These apparently opposite experimental results preclude making generalized conclusions with respect to the optimal SS-analog treatment modalities that might apply to patients with sst-positive neuroendocrine tumors. In addition, an escape from SS-analog treatment could, alternatively, involve an up-regulation of binding sites that do not recognize octreotide and/or an escape of tumor cells that do not express octreotide-responsive sst subtypes. In the majority of the above-mentioned studies, the precise mechanisms of changes in sst numbers were not studied in detail. Therefore, it remains to be established whether the changes in sst numbers at the cell surface are mediated via reduced sst gene transcription, decreased stability of sst mRNAs, via

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an increased intracellular breakdown of preexistent cellular SS receptors, or a combination of these events.

### D. Mechanisms of tachyphylaxis and resistance

*1. Homologous (down-)regulation of sst expression.* Although uncoupling from G proteins and internalization of SS receptors cannot be fully excluded as a potential cause for reduced sensitivity to long-term SS-analog treatment in patients with neuroendocrine tumors, other mechanisms are more likely to be involved. Down-regulation of cellular SS receptors may form a long-term cause of tachyphylaxis after continuous exposure of SS receptors to agonists. On the one hand, chronic exposure of cultured pituitary cells to relatively high concentrations of SS-14, SS-28, or SS-analogs reduces the number of sst on AtT20 and 7315b pituitary tumor cells (149, 151, 154, 155). On the other hand, an up-regulation of sst expression has been observed in $GH_4C_1$ or $GH_3$ rat pituitary tumor cells (156, 157). This up-regulation of SS receptors in $GH_4C_1$ or $GH_3$ was related to changes in sst gene expression, rather than changes in receptor affinity. In fact, in $GH_3$ cells, chronic exposure with SS induces an increase of $sst_1$, $sst_3$, $sst_4$, and $sst_5$ mRNA expression after 6–48 h of exposure, whereas $sst_2$ mRNA expression displayed a biphasic response, with an increase at 2 h, a decrease at 6 h, and finally normalization after 48 h (157). Therefore, agonist-induced down-regulation and/or up-regulation of sst expression is time dependent and cell type specific. In cells that do not express sst subtypes endogenously, but were transfected with the different sst subtype genes to overexpress the different sst subtypes, agonist exposure has differential effects, depending on the sst subtype investigated. Short-term (1 h) agonist exposure decreases SS-binding in CHO cells expressing the $sst_{2A}$ receptor (158, 159), whereas prolonged exposure (22 h) to the peptide induces an increased binding (54). SS binding in cells expressing $sst_3$ and $sst_5$ receptors was not affected by SS pretreatment, whereas $sst_4$ and $sst_1$ receptors were up-regulated (54). Whether these sst subtype-specific responses to agonist exposure also occur in human sst-positive tumors, which express multiple sst subtypes, remains to be established. To our knowledge, no such data are available at present, except for clinical data on responsiveness and the induction of tachyphylaxis to SS-analog therapy (see *Section III.B.2*). Apart from agonist-induced changes in cell surface sst number, tachyphylaxis of responsiveness after chronic agonist exposure and/or resistance to SS-analog treatment may be induced by several other potential mechanisms as well. Such mechanisms include heterologous regulation of SS cell surface numbers, heterogeneous expression of SS receptors in human tumors, or sst gene mutations, and they are discussed in the following paragraphs.

*2. Heterologous regulation of sst expression.* Apart from homologous regulation of sst expression (*Section III.D.1*), heterologous up- and down-regulation of SS receptors on normal and tumorous cells has been demonstrated as well. Glucocorticoids down-regulate sst numbers in $GH_4C_1$ rat pituitary tumor cells. In these cells, both cortisol and dexamethasone reduce the specific binding of $[^{125}I\text{-}Tyr^1]$SS-14 by 20% and 40%, respectively (160), probably via the inhibition of *de novo* protein synthesis. Moreover, sst subtype expression in $GH_4C_1$ cells is differentially regulated by glucocorticoids. Short-term incubation for 2 h with dexamethasone increases $sst_1$ and $sst_2$ mRNA levels, whereas $sst_3$ mRNA levels were unchanged. On the other hand, prolonged exposure (2 d) with dexamethasone induced a reduction in $sst_1$ and $sst_2$ mRNA levels and a dramatic up-regulation of $sst_3$ mRNA levels. Nuclear run-on assays showed that the changes in $sst_1$ and $sst_2$ mRNA levels were associated with changes in sst gene transcription rate (161). Indirect clinical evidence for the *in vivo* down-regulation of tumoral SS receptors by glucocorticoids was obtained from the observation that in five patients with untreated Cushings' disease, octreotide did not inhibit basal or CRH-stimulated ACTH levels and did not influence cortisol levels. *In vitro*, however, octreotide inhibited CRH-stimulated ACTH secretion by human corticotroph adenoma cultures, whereas this inhibitory effect was abolished by hydrocortisone pretreatment (162). Estrogens have been shown to stimulate sst expression in pituitary (tumor) cells (163–165) *in vitro* and *in vivo*. Chronic estrogen treatment up-regulates $sst_2$ receptor mRNA expression in the anterior pituitary gland *in vivo* (166). Considerably less information is available regarding the heterologous regulation of sst expression in nonpituitary-derived cell systems. In breast cancer cell lines, estrogen stimulates steady state mRNA levels (167). A 5.3-kilobase pairs (kb) 5′-flanking region of the $hsst_2$ gene, lacking TATA and CCAAT boxes, is the active promotor in estrogen receptor-positive breast cancer cell lines (168). In agreement with these observations, Kimura *et al.* (169) recently demonstrated that estrogen regulated promotor activity of a 5-kb 5′-untranslated region of the rat $sst_2$ gene, lacking TATA and CCAAT boxes (169). In concordance with the findings in pituitary-derived cells, dexamethasone may cause down-regulation of sst numbers without changing receptor affinity in AR42J rat pancreatic acinar carcinoma cells (170). Thyroid hormones may regulate sst expression as well. In TtT-97 tumors, which represent an *in vivo* murine thyrotropic model not expressing any sst subtype mRNA or protein, thyroid hormone treatment induces specific up-regulation of $sst_1$ and $sst_5$ mRNAs and high affinity sst binding sites in the tumors (171). Taken together, these data demonstrate that sst subtype expression can be influenced by different steroids and hormones in a time-specific and receptor subtype-specific manner. It is not established, however, whether treatment of patients with, for example, glucocorticoids or antiestrogens may influence sst expression and thus responsiveness to SS *in vivo* as well. Apart from a regulatory effect of glucocorticoids and estrogens on sst expression, it is also likely that such agents directly influence the responsiveness of tumor cells to SS agonists. Indeed, breast cancer cells have been shown to respond better to the cytotoxic effect of octreotide in the presence of the antiestrogen tamoxifen (172).

*3. Resistance to SS agonists*

*a. Heterogeneity of tumoral sst expression.* Certain subgroups of human sst-positive tumors express sst subtypes on the basis of their differential binding of SS and SS-analogs. The sst autoradiographic studies showed the absence of binding of $[^{125}I\text{-}Tyr^3]$octreotide in a small subgroup of human insu-

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linomas, carcinoids, pituitary adenomas, and meningiomas, in 50% of MTCs, and in all sst-positive ovarian cancers, whereas in the same tumors binding sites for iodinated-[$Tyr^{11}$]SS-14 or [LTT]SS-28 were present (23–25). This differential binding between octreotide on the one hand and SS-14/SS-28 ligands on the other hand, in insulinomas and other subgroups of sst-positive tumors, suggests that resistance to octapeptide SS-analogs may be due to the absence of specific sst subtypes that bind these analogs with high affinity, but also indicates that novel sst subtype-selective analogs can be developed for the treatment of patients with tumors carrying sst of this particular subtype(s). Although certain human sst-positive tumors lack particular sst subtypes with high affinity for octapeptide SS-analogs (Table 1), some tumors have been demonstrated to express a nonhomogenous distribution of SS receptors (23–25). A nonhomogenous distribution of sst has been found in a subset (3 of 10) of human GH-secreting pituitary adenomas (173), as well as in rare cases of carcinoid tumors (23). Moreover, in more than 50% of breast cancer specimens, sst expression displayed a nonhomogenous distribution, *i.e.*, both sst-positive and sst-negative tumor regions within individual sst-positive tumors (174). One rare case of a human carcinoid tumor has been described in which $sst_1$ and $sst_2$ mRNA were clearly localized in different tumor regions (28). In such cases, resistance to SS-analog therapy, after an initial response, may be due to the outgrowth of sst ($sst_2$)-negative tumor cell clones, which in fact may still express sst, albeit of the subtype to which the current generation of octapeptide SS-analogs do not bind.

*b. SS receptor gene mutations.* To date, relatively few data are available with respect to sst-gene mutations leading to a loss of sst function. One study addressed this issue so far in COR-L103 small cell lung cancer cells (175). Sequence analysis of the $sst_2$ gene demonstrated a point mutation in codon 188 of TGG for tryptophan to TGA for a stop codon causing a loss of 182 C-terminal amino acid residues in $sst_2$, resulting in the absence of $sst_2$ expression in the plasma membrane of COR-L103 cells. The nucleotide sequences of the $sst_3$ and $sst_4$ genes, which were also expressed in these cells, were normal. In a series of 19 human GH-secreting pituitary adenomas with variable sensitivity to SS-analog treatment *in vivo*, the $sst_2$ and $sst_5$ genes were found to possess intact coding sequences (176). Moreover, no mutations affecting the $sst_2$ protein were detected in a series of 15 GH-secreting pituitary adenomas (177). These data suggest that mutations in these sst subtypes do not form the basis for resistance of tumoral GH secretion to SS-analogs. Ballare *et al.* (178) recently described a germ line mutation (Arg240Trp) in the $sst_5$ gene in an acromegalic patient resistant to SS-analog treatment. This mutation results in decreased sensitivity to the inhibitory effect of SS on adenylate cyclase activity, whereas cells expressing the mutant $sst_5$ displayed increased proliferation and increased MAPK activity, compared with wt cells. These data suggest that this mutation in $sst_5$ abrogated the antiproliferative action by SS and activated mitogenic pathways. Nevertheless, such mutations appear to be very rare. Finally, in none of a series of 43 neuroblastoma tumors were mutations in the $sst_2$ gene detected by PCR-based single-stranded conformation polymorphism/heteroduplex analysis (179). Mutations in other sst subtypes that may cause this resistance cannot be excluded, however. Moreover, other causes such as sst density and/or the above-discussed mechanisms of resistance (summarized in Table 4) may play a role as well.

*c. Miscellaneous potential causes of resistance to SS-analogs.* Antibodies to octreotide that develop in patients treated with this analog (138, 180–182) seem not to be an important cause of escape from therapy with SS-analogs, because continued efficacy of octreotide treatment has been documented in two acromegalic patients who had antibodies to octreotide (181). G protein mutations, particularly mutations in $G_s\alpha$, have been shown to be associated with overproduction of hormones by pituitary-derived hormones, as well as with pituitary hyperplasia (183). In a subgroup of patients with GH-secreting pituitary adenomas, high basal adenylyl cyclase activity and poor responsiveness to stimulatory agents such as GH-releasing hormone suggested constitutive activation of the adenylyl cyclase cascade in the tumor cells. A considerable number of these tumors indeed contained an activating mutation in $G_s\alpha$ (183), which correlates with a higher sensitivity to SS agonists. An increase in $sst_2$ mRNA does not seem to account for this increased sensitivity (184). However, mutations in inhibitory G proteins are rare, and mutations in $G_i2\alpha$, to which $sst_2$ is capable of associating (185), have only been described in small numbers of adrenal cortical tumors (27%) and ovarian tumors (30%; Ref. 186). It appears therefore, that resistance to SS-analog therapy due to a mutation in inhibitory G proteins coupled to sst is not very likely to occur.

### *E. New developments*

As described in *Section III.D.3*, one of the causes for resistance to therapy with the current generation of octapeptide SS-analogs may be the absence or low expression of $sst_2$ receptors by the tumor cells. The question then arises: What might be the role of other sst subtypes as a target for therapy with novel SS-analogs? Functional evidence for the existence of sst subtypes comes from studies using human fetal pituitary cell cultures in which SS regulates GH and TSH secretion by both $sst_2$ and $sst_5$, and PRL secretion mainly by $sst_2$ (74). In recent years, many new sst selective analogs have been synthesized. Using primary cultures of human GH-secreting pituitary adenomas, Melmed and co-workers (75) demonstrated that combinations of $sst_2$- and $sst_5$-selective

TABLE 4. Potential mechanisms of tachyphylaxis and resistance to SS-analog therapy in patients with sst-positive tumors

1. Down-regulation: decrease in the number and/or affinity of SS receptors
2. Desensitization: decrease in responsiveness due to receptor uncoupling from second messenger activation
3. Nonhomogeneous expression of SS receptors in tumors: outgrowth of sst-negative cell clones
4. Resistance due to the absence of sst subtypes with high affinity for octapeptide SS-analogs
5. Resistance due to tachyphylaxis of the inhibitory effect of SS-analogs on indirect tumor growth-promoting mechanisms (*i.e.*, GH or gastrin secretion)
6. Mutations in sst genes leading to absence of functional receptor proteins

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compounds decreased GH secretion significantly more than the single compounds alone. In addition, in PRL-secreting primary tumor cell cultures, PRL secretion was preferentially inhibited by $sst_5$-selective analogs, whereas $sst_2$-selective analogs were ineffective. Even more exciting are recent studies using a bispecific sst analog with high affinity binding to both $sst_2$ and $sst_5$ receptors. This compound, BIM-23244 (Table 1), was quite effective in inhibiting GH secretion *in vitro* by a series of five octreotide partially responsive tumors. These tumors turned out to have 9-fold lower $sst_2$ mRNA levels and approximately 7-fold higher $sst_5$ mRNA levels, compared with a group of octreotide-sensitive tumors. The same compound also inhibited PRL release by five mixed GH-PRL-secreting pituitary adenomas (187). Thus, apart from highly sst-selective analogs, there may be a place for new sst bispecific analogs in the treatment of pituitary adenomas resistant to $sst_2$ agonists. More recently, a SS peptidomimetic, named SOM230, with high affinity for $sst_1$, $sst_2$, $sst_3$, and $sst_5$ receptors (Table 1) has been shown to have a much higher efficacy in lowering normal plasma IGF-I levels in rats, compared with the effects of the $sst_2$-selective analog octreotide (188). Long-term (weeks to months) continuous, as well as discontinuous, treatment with octreotide in rats is known to result in a loss of the inhibitory effect of the drug on circulating GH and IGF-I levels (148, 149, 189). The potent inhibitory effect of SOM230 on IGF-I levels, showing no signs of loss of its inhibitory effect during a period of 126 d of continuous infusion, could be explained by a 40-fold increase in the affinity for $sst_5$ receptors, as compared with octreotide in combination with the key role that $sst_5$ plays in controlling GH release (75, 187). SOM230 has a very long terminal elimination half-life of 23 h in rats, compared with octreotide (2 h), and no obvious adverse side effects, including changes in glucose levels, over the 126-d period of treatment, and it is currently under evaluation in phase I trials (188). Moreover, sst subtypes may form homo- or heterodimers (56, 58) or may heterodimerize with other G protein-coupled receptors such as the dopamine D2 receptor (60) or the opioid receptor MOR-1 (59), resulting in a novel receptor state with properties distinct from the individual receptors in terms of enhanced internalization, reduced agonist-induced desensitization, and functional activity. These new fundamental insights into receptor function will help us to explain the observed differences in the development of tachyphylaxis not only between patients with different tumor types, but also among patients with the same type of neuroendocrine tumor but with different sst subtype expression patterns. It is a challenge to evaluate whether these new bispecific or more universal SS-analogs are indeed effective in tumors resistant to the current clinically available compounds as octreotide and lanreotide, as well as to investigate whether such new compounds can prevent neuroendocrine tumors from tachyphylaxis to treatment. Apart from new analogs with a broader sst binding profile, a hybrid SS-dopamine molecule has also been recently synthesized. This molecule, BIM-23A387, retained high affinity binding to both $sst_2$ and D2 receptors and had a tremendous enhanced potency on GH and PRL release by primary cultures of human pituitary adenoma cells, compared with $sst_2$- and D2-specific analogs, alone or in combination (190). This significant enhanced potency, however, could not be explained on the basis of the binding affinity of the compounds for $sst_2$ and D2 receptors (190). The mechanism by which this molecule exerts its potent action is unknown but strengthens the observations that processes like heterodimerization of receptors indeed have functional implications.

### *F. Conclusions*

The induction of tachyphylaxis of responsiveness to SS-agonists has been demonstrated in a variety of sst-positive cell systems. The time-frame of the occurrence of tachyphylaxis *in vivo* on normal hormone secretion is relatively rapid (hours to days), whereas escape from therapy with SS-analogs in patients with sst-positive tumors or in experimental models of sst-positive tumors generally occurs after prolonged exposure to SS agonists (weeks to years). This relative late induction of tachyphylaxis of responsiveness suggests that sst down-regulation, rather than rapid processes like G protein uncoupling and/or receptor internalization are involved. Moreover, escape from SS-analog therapy could involve the outgrowth of tumor cell clones lacking the expression of sst subtypes to which the currently clinically used octapeptide analogs bind with high affinity. The development of novel sst subtype-selective and nonselective analogs, as well as chimeric compounds, could be of interest as potential new treatment modalities for resistant tumors. The only group of tumors that show no signs of desensitization to treatment with SS-analogs are GH-secreting pituitary adenomas. In SS-analog-sensitive patients with GH-secreting pituitary adenomas, circulating GH and IGF-I concentrations can be effectively suppressed, even during many years of treatment with these compounds. The underlying mechanisms for this difference in developing tachyphylaxis to SS-analog treatment between GH-secreting pituitary adenomas on the one hand, and other types of neuroendocrine tumors on the other hand, have not yet been elucidated but could involve the differential expression of sst subtypes, a tissue-specific desensitization, and/or down-regulation of sst subtypes, or alternatively, tissue-specific up-regulation of SS-analog responsive sst subtypes by prolonged agonist treatment resulting in continued responsiveness.

In conclusion, clinical observations clearly demonstrate tachyphylaxis and/or resistance to SS-analog treatment in patients with neuroendocrine tumors, but the direct fundamental evidence explaining the mechanisms involved is currently weak.

## IV. Summary

During the past decade, novel insights into the physiological and pathophysiological role of SS and its receptors have been developed. Although in the mid-1980s it was debated whether or not SS was internalized by sst-expressing cells, recent studies have now clearly demonstrated that SS and SS-analogs are efficiently internalized via a rapid process of agonist-induced receptor-mediated endocytosis. Moreover, in 1989 the technique of sst scintigraphy to visualize sst-positive tumors in humans was developed, and the concept of the radiotherapeutic use of radioisotope-coupled SS-

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analogs, *i.e.*, peptide receptor radionuclide therapy, was introduced. Finally, in the beginning of the 1990s, five sst subtypes were cloned and characterized, and the expression of these subtypes has been studied in both normal and tumoral sst-expressing tissues. Taking these discoveries together, several new questions can be raised. These include: 1) Which sst subtypes that are expressed in human sst-positive tumors determine (un)responsiveness to octapeptide SS-analogs such as octreotide or lanreotide, and is there a role for novel sst subtype selective SS-analogs? 2) Which sst subtypes are involved in receptor-mediated endocytosis of radiolabeled SS-analogs and form the basis for targeted radiotherapy or chemotherapy using SS-analogs coupled with radioisotopes or chemotherapeutic compounds, respectively? and 3) What is the role of the individual sst subtypes in determining responsiveness, as well as tachyphylaxis of responsiveness of sst-positive cells upon agonist exposure? In this review, the current knowledge of the clinical consequences of agonist-induced sst internalization for treatment for sst-targeted radiotherapy or chemotherapy are discussed, as well as the different mechanisms that could play a role in tachyphylaxis and/or resistance to SS-analog therapy in patients with neuroendocrine tumors.

The individual sst subtypes differentially internalize SS-(analogs). The $sst_1$ receptors show low agonist-induced internalization, whereas $sst_2$, $sst_3$, $sst_4$, and $sst_5$ are more efficient in this respect. The predominant expression of $sst_2$ receptors in most human sst-positive neuroendocrine tumors and the efficiency of $sst_2$ receptors to undergo agonist-induced internalization is very important for the radiotherapeutic application of radiolabeled octapeptide SS-analogs. *In vitro* studies have demonstrated that $sst_2$-expressing tumor cell lines, as well as primary cultures of human tumors, internalize radiolabeled SS-analogs such as [$^{111}$In-DTPA$^0$]octreotide, [$^{90}$Y-DOTA$^0$,Tyr$^3$]octreotide, and [$^{90}$Y-DOTA]lanreotide. Preclinical studies using experimental tumor models have now demonstrated that tumor growth can be inhibited by administration of radiopharmaceutical compounds as [$^{111}$In-DTPA$^0$]octreotide and [$^{90}$Y-DOTA$^0$,Tyr$^3$]-octreotide. Clinical trials have already demonstrated promising effects using these radiopharmaceuticals, as well as of [$^{90}$Y-DOTA-lanreotide], on tumor size in patients with advanced sst-positive neuroendocrine tumors. Finally, the concept of targeted chemotherapy to deliver chemotherapeutic compounds selectively to sst-positive tumor cells, thereby reducing their toxicity, has now been validated using newly developed cytotoxic SS-analogs in experimental mouse and rat models of human pancreatic, breast, prostate, ovarian, and small cell lung cancer.

The presence of $sst_2$ receptors in tumors is a prerequisite for sensitivity of inhibition of tumor-related hormonal hypersecretion to treatment with octapeptide SS-analogs. The successful clinical application of SS-analogs such as octreotide and lanreotide in the treatment of hormonal hypersecretion in patients with GH-secreting pituitary adenomas and islet cell or carcinoid tumors is caused by the predominant expression of $sst_2$ receptors in these tumors. On the other hand, novel sst subtype-selective analogs, as well as bispecific and more universal agonists, have been synthesized now and were demonstrated to be effective in the *in vitro* inhibition of hormone secretion of sst-positive tumors that do not express $sst_2$ receptors. Patients with sst-positive tumors also show considerable variability in their responsiveness to treatment with SS-analogs. Patients with GH-secreting pituitary adenomas do not show desensitization to treatment with SS-analogs, whereas patients with islet cell or carcinoid tumors often demonstrate tachyphylaxis to treatment. The occurrence of tachyphylaxis upon treatment with SS-analogs is highly variable. Some patients escape very rapidly, whereas others show tachyphylaxis only after several years of treatment. Nevertheless, despite the increasing fundamental knowledge on the role of individual sst subtypes in agonist-induced internalization and/or desensitization of sst subtypes, as well as in agonist-induced, sst subtype-specific regulation of sst expression and receptor homo- and heterodimerization, the direct fundamental evidence for the observed differences between patients with neuroendocrine tumors in the development of tachyphylaxis to SS-analogs is currently weak and requires further studies.

## Acknowledgments

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