The functional role of somatostatin receptor subtypes in pituitary adenomas

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The Functional Role of Somatostatin Receptor Subtypes in Pituitary Adenomas

De Functionele Betekenis van Somatostatine Receptor Subtypen in Hypofyse Adenomen

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Chapter I

INTRODUCTION

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Somatostatin and somatostatin receptors

Over three decades have now elapsed since Brazeau and Guillemin originally detected a somatotropin release-inhibiting factor (SRIF) by chance during studies of the distribution of growth hormone-releasing factor in the hypothalamus of rats (1). This peptide, called somatostatin because of its supposed specific function, i.e. the inhibition of somatotropin [growth hormone (GH)] release, proved to be a cyclic peptide consisting out of 14 amino acids (Fig. 1). Higher molecular weights forms of SRIF immunoreactivity have been reported including a 28-amino acid

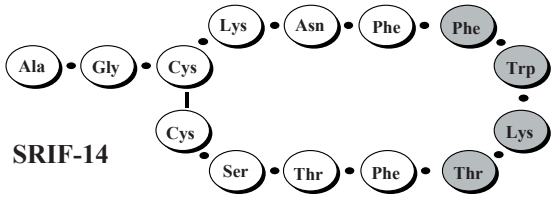

Figure 1. Amino acid sequence of the natural somatostatin peptide SRIF-14.

polypeptide that corresponds to SRIF with a NH_2-terminal extension of 14 amino acids. Isolation and characterization of complementary DNA (cDNA) clones encoding SRIF indicated that SRIF, like other polypeptides, is derived from a larger precursor by proteolytic processing. The primary translation product of SRIF messenger RNA (mRNA) is a 116-amino acid molecule, preprosomatostatin, subsequently processed in a 92-amino acid prosomatostatin which by itself undergoes proteolytic processing, thereby generating the biologically active forms SRIF-14 and SRIF-28. The production of SRIF occurs in concentrated numbers of SRIF-producing cells throughout the central and peripheral nervous systems, in the endocrine pancreas, in the gut and in limited cell numbers distributed over the thyroid, the retina, adrenals, submandibular glands, kidneys, prostate and placenta. SRIF modulates neurotransmission in the central nervous system (as a neurotransmitter), regulates the release of GH and thyrotropin (TSH) from the anterior pituitary gland (as a neurohormone) and has a regulatory role in the gastrointestinal tract, as well as in the exocrine and endocrine pancreas. When synthesized in and re-

leased by endocrine and nerve cells, SRIF acts in an autocrine, paracrine, or neuronal regulatory manner to inhibit glandular secretion, neurotransmission, smooth muscle contractility and absorption of nutrients (2, 3).

Somatostatin receptor subtypes

The various actions of SRIF are mediated through specific membrane receptors. Five subtypes of the human SRIF receptor (sst) have been cloned and characterized (4, 5). Genes for $sst_{1,3,4,5}$ lack classical introns. The sst_2 gene displays a cryptic intron at the 3' end of the coding segment, which gives rise to two splice variants, a long (sst_{2A}) form and a shorter (sst_{2B}) form, which differ only in the length of their cytoplasmic tail (6, 7). Even though Northern blotting has detected two bands of 2.3 kb and 8.0 kb size-length in human tissues (6, 8, 9), detailed evidence for the (functional) expression of the short sst_{2B} form in humans is still lacking. The sst subtypes, which are identical in 42 to 60 percent of their amino acid sequences, belong to a superfamily of seven α helical transmembrane segments typical of guanine nucleotide binding (G) protein coupled receptors (GPCR; Fig. 2). All subtypes share a coupling to the

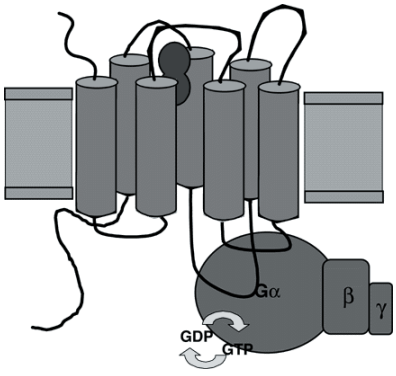

Figure 2. Structure and schematic orientation of the SRIF receptor within the plasma membrane encountering seven transmembrane-spanning α-helical domains connected by short loops, having an N-terminal extracellular domain and a C-terminal intracellular domain.

second messenger system known to be activated upon SRIF binding to its receptors. Hydrophobic and charged amino acids within transmembrane domains 3, 6 and 7 are important for the interaction with the ligand

(10, 11). However, the extracellular loop 2, between domains 4 and 5 may also be involved (12-14). The subsequent conformational change of the receptor leads to activation of an associated heterotrimeric G-protein complex (consisting of α-, β- and γ-subunits) and exchange of GTP for GDP on the α-subunit. The common effect of the five sst subtypes is a reduction in intracellular cyclic adenosine monophosphate (cAMP) and Ca^{++} as well as an activation of protein phosphatases [Fig. 3; (15)]. The final pathway, and hence effect on cellular function, will vary, depending on the specific sst and SRIF ligand involved. Inhibition of cell secretion may be achieved through several intracellular effector pathways: (1) inhibition of adenylyl cyclase activity via inhibitory G proteins (Gαi), which are pertussis toxin sensitive, thereby reducing intracellular cAMP levels, (2) reduction in intracellular Ca^{2+} owing to activation and hyperpolarisation of K^+ channels as well as inhibition of the normal depolarization-induced Ca^{++} influx via voltage-sensitive Ca^{++} channels (16). Both a reduction on intracellular cAMP and Ca^{++} result in inhibition of secretion. Stimulation of protein tyrosine phosphatases and inhibition of MAP kinase activity (17) by sst are thought to be involved in anti-proliferative effects of SRIF. Less prominent signaling pathways include inhibition of Na^+-H^+ exchanger and activation of phospholipase A and C (2, 18).

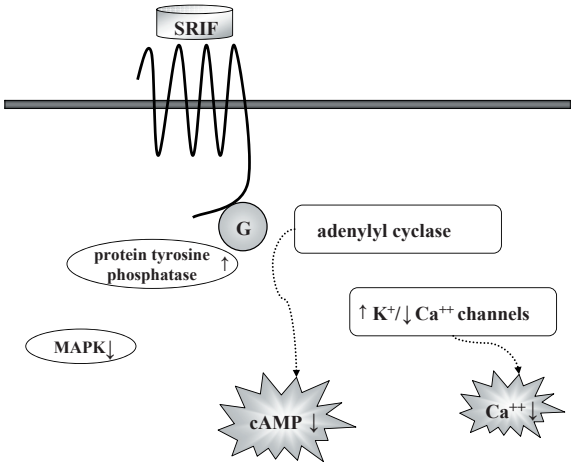

Figure 3. Schematic representation of SRIF receptor signaling pathways leading to inhibition of secretion and cell proliferation.

Distribution of somatostatin receptor subtypes

Classical SRIF-target tissues such as the central nervous system, the anterior pituitary gland and the pancreas, express multiple sst subtypes (Table I) (19). The adult human pituitary gland expresses sst_1, sst_2, sst_3 and sst_5 mRNAs, but not sst_4 mRNA. As determined by immunohistochemistry, the human pancreatic islet cells express all five sst subtype proteins (20). Neuroendocrine tumours frequently express a high density of SRIF receptors.

Table I. Distribution pattern of SRIF receptors in humans.

	SRIF receptor subtype				
	sst_1	sst_2	sst_3	sst_4	sst_5
Brain	+	+	+	+	+
Pituitary	+	+	+		+
Stomach	+	+	+	+	+
Liver	+	+			
Pancreas	+	+	+	+	+
Kidney	+	+			
Lung	+	+		+	
Intestine	+	+			+
Spleen		+			
Thymus	+	+	+		
Uterus		+			
Thyroid			+		+
Prostate	+	+	+		
Adrenal		+			+

The SRIF-receptor subtype distribution (by in situ hybridistation, immunohistochemistry, RT-PCR analysis, Northern Blotting) in normal tissues has been extensively reviewed. For further details see Refs. (2, 5, 9, 15, 19, 20, 25, 26).

Sst-expressing tumours include pituitary adenomas, carcinoids, islet cell tumours, paragangliomas, pheochromocytomas, small cell lung cancers, medullary thyroid tumours but also breast cancers, prostate cancers and malignant lymphomas (21). The majority of human sst-positive tumours simultaneously express multiple sst subtypes, which can be different between tumour types but also among the same tumour types. The predominant expression of sst_2 receptors forms the basis for the successful clinical application of the currently available clinical SRIF-analogs. The presence of sst_2 is a prerequisite for sensitivity of inhibition of tumour-related hormonal hypersecretion to treatment with octapeptide SRIF-analogs, as has been extensively demonstrated in patients with growth hormone (GH)-secreting pituitary adenomas and islet cell or carcinoid tumours (22, 23).

Somatostatin receptor physiology

Although the acute administration of SRIF produces a large number of inhibitory effects, the initial responses diminishes with continued exposure to the peptide. The ability to regulate their responsiveness to continued agonist-exposure is a common property of many GPCR's. Such agonist-induced regulation typically involves receptor desensitization due to uncoupling from G proteins as well as receptor internalization and receptor degradation (2). Since the cloning of the five sst subtypes, as already described previously, the involvement of the individual sst subtypes in the process of receptor-mediated internalization of SRIF has been extensively investigated (2, 21, 24-26), and it should be taken into account that differences have been reported between human and rat sst subtypes with respect to their dynamics of agonist-induced internalization. In general, the mechanism and route of internalization of sst-agonist complexes involves aggregation of the hormone-receptor complex in specialized areas of the membrane, followed by internalization of the complex via clathrin-coated, as well as uncoated, pits. Subsequently, fusion of these vesicles with lysosomes occurs, resulting in hormone degradation or receptor recycling to the cell surface. The sst subtypes differentially internalise SRIF and SRIF-analogs. Using CHO-K1 cells, stably expressing

one of the five sst subtypes, agonist dependent internalization of [^{125}I]LTT SRIF-28 ligand in a time- and temperature dependent manner, has been demonstrated (27). The human sst_1 receptor showed low agonist induced internalization (4%), whereas 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%). Furthermore, binding of SRIF to monomeric cell-surface sst_1 and sst_5 triggers both homo- and hetero-sst dimerization. The sst_1-sst_5 heterodimers show enhanced ligand-receptor binding affinity likely the result of receptor subtype modification. The functional significance of receptor dimerization, restricted to only some sst's, varies according to the receptors involved; human sst_5 forms heterodimers with sst_1, but not with sst_4 (28). Using the selective sst_1 ligand ^{125}I-SCH288, no internalization of sst_1 was observed. However, when sst_1 receptors where co-transfected with a c-tail deletion mutant of sst_5, a slight but significant internalization of ^{125}I-SCH288 at 60 minutes was observed, suggesting that processes like functional heterodimerization of sst subtypes can determine internalization of sst subtypes (29). Interestingly, Stroh and coworkers elegantly demonstrated by the use of biochemical, confocal and microscopic techniques, that a constant population of functional rat sst_5 receptors is maintained at the cell surface at all times through a process of receptor recycling and recruitment of intracellular sst_5 proteins (30). They proposed that these mechanisms might protect sst_5 from long-term desensitization. Furthermore, rat sst_{2A} and sst_3 are able to form homodimers as well. Both sst_2 and sst_3 homodimers underwent agonist induced endocytosis, but the heterodimer of sst_{2a} and sst_3, both being over-expressed in HEK293 cells, dissociated at the cell membrane and only sst_2 underwent agonist induced endocytosis combined with loss of sst_3 function (31). In addition to this communication between receptor subtypes within the same GPCR family, rat sst_{2A} receptor heterodimerization with the μ-opioid receptor (MOR1) has also been demonstrated (32). The sst_{2A}-MOR1 heterodimers did not significantly alter the ligand binding or coupling properties but promoted cross-modulation of phosphorylation, internalization and desensitization of these receptors. Finally, studies using bioluminescence resonance energy transfer assays

(BRET) on living cells, have also indicated that hetero-oligomerization of human sst_5 and the Dopamine D2 receptor occurs following to agonist binding (33). The "new receptor" formed by the heterooligomerization of the D2 receptor and sst_5 appeared pharmacologically distinct from its receptor homodimers, as it was characterized by a much greater affinity for binding both dopamine and SRIF-agonists, directly associated with enhanced G-protein and effector coupling to adenylyl cyclase. Recently, the first data on heterodimerizaton *in vivo* has been demonstrated. In normal rat astrocytes, sst_2 and D_2 receptors formed hetero-oligomeric complexes, displaying properties distinct from the native receptors (34).

Acromegaly

Acromegaly is predominantly caused by a GH-secreting pituitary adenoma, resulting in high circulating GH and insulin-like growth factor I (IGF-I) hormone concentrations. Over the years, a triad of therapeutical options has been formed by surgery, irradiation and medical treatment (35). These treatment modules had the aim to induce tumor shrinkage and normalisation of GH and IGF-I levels, thereby reducing the risk of long term complications including the development of malignant neoplasms, cardio- and cerebrovascular disease, respiratory and metabolic dysfunction (36). Effective treatment reduces the 2-3 times raised mortality rate for acromegalics (35, 37). Epidemiological studies have confirmed the elevated mortality rate associated with acromegaly and the ability of effective GH-lowering treatment to improve mortality rate (36-38). While surgical resection has been the treatment of choice in most GH-secreting pituitary tumours, there has been increasing recognition that curative resection is not routinely achieved, even by the most experienced neurosurgeon. Moreover, a significant number of pituitary adenomas are already invasive adenomas at the time of diagnosis, a finding that further undermines the success of surgical management (39-41). External radiotherapy can be an effective adjuvant therapy, but often it takes several years to normalise GH and IGF-I levels, there is a risk for the development of anterior hypopituitarism and in the long term cerebral damage cannot be ex-

cluded (42). The clinical introduction of stable long acting SRIF analogs, Octreotide (OCT) and Lanreotide (Fig. 4), in the early 1980s added a new dimension to the treatment of acromegaly (43, 44). OCT and Lanreotide form a safe and effective medical therapeutic modality for acromegaly, mimicking the action of the native hypothalamic peptide SRIF as the principal negative regulator of GH secretion by pituitary somatotrophs (1).

Biochemical efficacy of currently available SRIF-analogs

Intravenous administration of SRIF results in a marked inhibition of GH release in most acromegalic patients (45, 46). The short half-life of SRIF and its subsequent need for intravenous administration, however, as well as the post-infusion hypersecretion of GH, insulin and glucagon, has rendered the native peptide impractical for therapeutic use (47). During the 1980s, SRIF-analogs were clinically introduced (43, 48). These short, stable synthetic octapeptide SRIF-analogs appeared not to have these disadvantages and were administered in acromegalic patients to assess their role as a novel treatment option in acromegaly. Indeed, after the first reports that demonstrated the long-acting inhibitory effect of OCT on plasma GH level, as well as a rapid amelioration of the clinical signs and symptoms, twenty years of endocrine practice and science has turned OCT and Lanreotide (Fig.4) into a widely accepted medical treatment option for acromegaly (22).

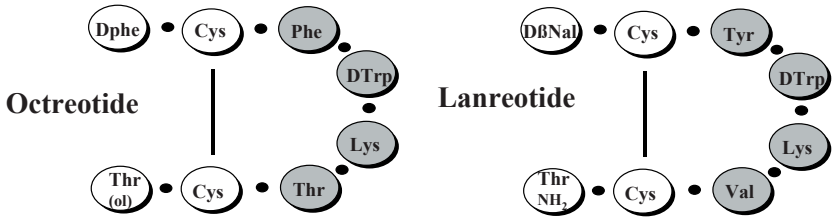

Figure 4. Amino acid sequence of the two current clinically available SRIF-analogs Octreotide and Lanreotide.

Biochemical control

The current clinically available octapeptide SRIF-analogs have been consistently shown to be able to reduce hormonal hypersecretion and to

normalize IGF-I Ievels in a significant proportion of treated patients. Recently, Freda, summarizing the literature data on this topic (49), showed the achievement of safe GH levels in 56% of patients treated with Sandostatin-LAR (OCT incorporated into microspheres of a biodegradable polymer that results into therapeutical blood concentrations of the peptide for 24-42 days) and in 49% of those treated with Lanreotide 30 mg. The respective figures for IGF-I normalization were 66% and 48%. However, a large majority of the patients enrolled in these studies, were preselected for SRIF-analog responsiveness (49). The observation that SRIF-analogs successfully achieve long-term biochemical cure when used as secondary or adjuvant therapy (50, 51), led to investigations of the use of SRIF-analogs as primary therapy for acromegaly. Therefore, several studies were initiated in which newly diagnosed patients were treated with SRIF-analogs. To date, efficacy numbers have been reported for normalizing GH (43-79%) and IGF-I (53 and 68%), which are comparable with efficacy numbers for adjuvant SRIF-analog therapy (52-58). Furthermore, Ayuk and co-workers retrospectively re-analysed GH and IGF-I data from a large multicenter European Study in which patients' biochemical response to treatment with Sandostatin-LAR as primary or secondary therapy was evaluated after 12 months of treatment (59). To eliminate preselection bias due to prior debulking of tumor mass, GH and IGF-I levels taken from 91 patients at diagnosis were used as baseline values. After 48 weeks of treatment, Sandostatin-LAR was equally effective as primary therapy in acromegalic patients as well as in patients previously treated with surgery and/or radiotherapy (59). Several recent reports have evaluated Lanreotide Autogel, the newest long acting SRIF-analog preparation, for the treatment of acromegaly. After 1 year of treatment with titrated doses of Lanreotide Autogel (dose increased if GH >2.5 μg/L, or decreased if GH < 1 μg/L with normal IGF-I), mean GH and IGF-I concentration was controlled in 68% of patients (60). In addition, an open label multicenter study demonstrated that Lanreotide Autogel, in 25 acromegalic patients, appeared as efficacious as compared with the patients' previous biochemical efficacy numbers for Sandostatin-LAR (61). SRIF-analog therapy, in general, is well tolerated by most patients. Ad-

verse effects are comparable among the available (long- and short acting) SRIF-analogs and do not limit treatment. The most common side effects are gastro-intestinal, *i.e.* nausea, vomiting, diarrhea and abdominal discomfort (49). As SRIF-analogs modulate the secretion of insulin and glucagon combined via sst expression within the endocrine pancreas, reduced glucose tolerance and even overt hyperglycaemia were initially expected during long-term therapy. On the contrary, the effects on glucose homeostasis appeared to be minor and a mild deterioration occurred only in those without impaired glucose tolerance (22).

Sst subtype expression & SRIF-analog efficacy

Although the clinical introduction of SRIF-analogs has nowadays emerged into their use as reference drug for the medical treatment of acromegaly, still, no more than approximately two thirds of cases of persistent acromegaly respond satisfactory to these agents (62). Human GH-secreting pituitary adenomas express multiple SRIF receptor subtypes. Sst_2 and sst_5 receptors are the predominantly expressed SRIF receptor subtypes, both at the mRNA (63, 64) and the protein level (19). Several studies reported a variable sst_2 mRNA expression and a relative high expression of sst_5 (63-65). Sst_2 seems a predominant receptor in determining the inhibitory effect of OCT or lanreotide on circulating GH release in acromegalic patients. Sst_2 mRNA expression in GH-secreting pituitary adenomas shows a positive correlation with the in vivo GH suppression induced by an acute test using a single injection of 200µg OCT (65). Moreover, recently it has become clear that apart from sst_2, sst_5 receptors play an important role in regulating GH secretion by human GH-secreting pituitary adenoma cells as well. In this respect the regulation of fetal human GH secretion (66) is similar to that in human GH-secreting pituitary adenomas. In primary cultures of human GH-secreting pituitary adenomas, new SRIF-analogs with enhanced sst_2 binding affinities inhibit GH secretion more potently compared to the clinically used octapeptide SRIF-analogs OCT and lanreotide. In addition, some adenomas show a better response to sst_2-specific analogs, whereas in others sst_5-specific analogs are more potent

in suppressing GH release (67). Moreover, the combined activation of sst_2 and sst_5 results in additive inhibitory effects on GH secretion. Interestingly, the sst_5 preferential analog BIM-23268 inhibited GH release in only 7 of 15 cases, whereas, in agreement with the results of Shimon and coworkers (67), partial additive effects in suppressing GH release were found in OCT-partially responding cultures when the sst_2- and sst_5- specific compounds used in combination. Taken these data together, it can be concluded that sst_2 is the predominant receptor in regulating GH release by GH-secreting pituitary adenoma cells, whereas sst_5 receptors may mediate an inhibitory effect on GH secretion as well. The additive inhibitory effects on GH release following activation of both sst_2 and sst_5 are likely mediated via a functional association of both sst subtypes. Ren and coworkers (68) demonstrated in human fetal pituitary cell cultures that a sst_2 selective antagonist was capable of reversing the GH suppressive effects of sst_2/sst_5 biselective agonists, or that of sst_2 and sst_5 agonists in combination, indeed suggesting a functional interaction between both sst subtypes. In adenomas co-secreting GH and prolactin (PRL), PRL secretion is preferentially inhibited by sst_5-specific SRIF-analogs (63, 67). The observed additive GH-suppressive effect of activating both sst_2 and sst_5 also initiated the development of analogs with selectivity to multiple sst subtypes. One of these compounds, the sst_2- and sst_5- bispecific compound BIM-23244, indeed inhibits GH release in a subgroup of partially OCT-sensitive adenomas more potently compared with OCT. In this subgroup of adenomas sst_2 mRNA expression was nine-fold lower, and sst_5 mRNA expression approximately seven-fold higher than in the OCT-sensitive adenomas (65). These studies suggest that in tumors expressing a low sst_2 level and a high sst_5/sst_2 ratio, sst_5 is of increasing importance in regulating GH release (65). Another recently developed compound has a more universal sst binding profile. Lewis and coworkers succeeded in synthesising the multiligand SOM230, a stable SRIF-analog with more universal binding profile to sst subtypes, which displays a 30-, 5- and 40- times higher binding affinity to sst_1, sst_3 and sst_5 receptors, respectively, and 2.5 times lower affinity to sst_2, compared with OCT (69). By using an alanine scanning technology, essential functional groups of the SRIF peptide respon-

sible for the high binding affinity to all five sst subtypes were detected. Incorporation of four synthetic amino acids and two essential amino acids of SRIF into a stable cyclohexapeptide template resulted into SOM230. In rats, dogs and monkeys SOM230 potently and dose-dependently decreases GH and IGF-I levels more efficacious as compared with OCT (70, 71). Additional characteristics of SOM230 include a very favourable T½ of nearly 24 hours, as well as the preliminary evidence that glucose levels in rats and dogs remain normal during long-term administration (70). As already discussed, heterodimerization of sst_5 and dopamine D2 receptors (D2DR) seems to result in the formation of a novel receptor with enhanced biological activity (33). On the basis of these data, including the observation that combined SRIF-analog and dopamine agonist treatment inhibits GH hypersecretion in a significant proportion of acromegalic patients partially resistant to OCT or lanreotide (72), Saveanu et al. (73) recently studied the effects of BIM-23A387, which selectively binds with high affinity to sst_2 and D2DR receptors, on GH and PRL release by 11 cultured GH-secreting pituitary adenomas. In both OCT-sensitive, as well as in cultures showing partial responsiveness to OCT, the maximal inhibition of GH release induced by the individual sst_2 and D2DR analogs and by BIM-23A387 was similar. However, the mean IC_{50} for GH suppression by BIM-23A387 (0.2pM) was 50 times lower than that of the individual sst_2 and D2DR specific compounds. This enhanced potency of chimeric molecules, such as BIM-23A387, may therefore lead to potential novel medical treatment options in acromegalic patients.

Cushing's disease

The proposition that the pituitary gland is responsible for the clinical features of Cushing's disease was convincingly described for the first time in Harvey Cushing's classic monograph of 1932 (74). Much uncertainty remained at that time on the pathophysiologic mechanism of this disorder, yet the crucial clinical and pathological observations were made. Today it has been recognized that chronic overproduction of cortisol by hyperplastic adrenocortical glands is directly responsible for the clinical

features of Cushing's disease, a phenomenon which is primarily driven by pituitary adrenocorticotropin hormone (ACTH) hypersecretion (75).

Transsphenoidal surgery is the treatment of choice for pituitary-dependent Cushing's disease. Although transsphenoidal surgery allows cure of Cushing's disease, the reported success rates vary from 50 to almost 90% (76-80). If surgery fails radiotherapy (81) either alone or in combination with adrenolytic agents may be used (82-85). Unfortunately, none of the current treatment modalities ensure a full and permanent cure, as the rate of recurrence of the disease, depending on the criteria of initial cure, varies from 5 to 24% in the literature (81, 86-90). Therefore, physicians explored new medical strategies, preferably based on fundamental and (patho-) physiological pathways, in the hope of increasing the curation chance in this group of patients. Neuromodulatory agents, such as dopamine and SRIF, have been proposed to be of therapeutic interest in the medical treatment of Cushing's disease. Since the initial report showing that SRIF infusion induced a partial decrease of plasma ACTH in five patients with Nelson's syndrome (91), characterized by a rapidly growing ACTH-secreting pituitary tumor and skin pigmentation which develops in a minority of patients with Cushing's disease after bilateral adrenalectomy (92), there have been a few studies of its action in Cushing's disease and Nelson's syndrome using the SRIF-analog OCT. The conclusion of these various anecdotal reports is that the current clinically available SRIF-analog OCT is ineffective in treating Cushing's disease (93-95) (Table II).

SRIF in normal corticotrophs

Data with respect to sst subtype expression in normal mammalian corticotrophs are equivocal. In rat pituitary cells, co-localization of all five sst with ACTH expressing cells has been reported (96). In another study, sst_5 mRNA was found in 38% of normal corticotrophs, and sst_2 in only 3% (97), whereas Mezey and coworkers used double immunohistochemical techniques which demonstrated sst_2 in a large population and sst_5 in a small population of corticotrophs (98). Studies on the role of SRIF

and SRIF-receptors on ACTH release are equivocal as well, whereas it is generally accepted that sst_2 and sst_5 subtypes are mostly involved in the regulation of hormone (GH, PRL and TSH) release by human fetal anterior pituitary cells (66). No inhibitory effects of 1-100 nM SRIF were observed on basal and corticotropin releasing hormone (CRH)-stimulated ACTH release by cultured normal rat pituitary cells and pituitary halves (99, 100). In two studies, however, an inhibitory effect of 0.6-6

Table II. Role of SRIF and SRIF-analogs in Cushing's disease.

First author (yr; *ref. no*)	Patient no.	Compound	Effect on ACTH
		Cushing's disease	
Benker (1976; *107*)	1 (ADNX)	500µg SRIF / 60 min	-50%
Julesz (1980; *112*)	1 (ADNX)	250µg SRIF / 60 min	Decreased
Lamberts (1989; *93*)	3	100µg OCT	No effect
Ambrosi (1990; *95*)	2	500µg SRIF / 60 min	No effect
	5	100µg OCT	No effect
Stalla (1994; *94*)	5	200µg OCT / 180 min	No effect
		Other groups	
Tyrell (1975; *91*)	5 (Nelson)	500µg SRIF / 60 min	-40 to -70%
Benker (1976; *107*)	1 (Nelson)	500µg SRIF / 60 min	-50%
	1 (Addison)	500µg SRIF / 60 min	Slight decrease
Fehm (1976; *111*)	5 (ADINS)	500µg SRIF / 60 min	-44%
Julesz (1980; *112*)	1 (Nelson)	250µg SRIF / 60 min	Decreased
Lamberts (1989; *93*)	1 (Nelson)	2 yr 100µg OCT t.i.d.	Decreased
	2 (Nelson)	100µg OCT	Decreased
Ambrosi (1990; *95*)	5 (Addison)	500µg SRIF / 60 min	No effect
Petrini (1994; *114*)	1 (Nelson)	2 yr 100µg OCT t.i.d.	Decreased
Kelestimur (1996; *113*)	1 (Nelson)	100µg OCT	-46%

ADNX, adrenalectomized; ADINS, adrenal insufficiency of different origin; t.i.d., three times daily.

nM and 10 nM to 1 µM SRIF was reported on CRH and arginine vasopressin-stimulated ACTH release by cultured pituitary cells prepared

from long-term adrenalectomized rats (101) and by fresh quarters of pituitary glands (102), respectively. In addition, it was shown that basal and CRH-stimulated ACTH release by normal rat pituitary cells were insensitive to natural SRIF and OCT in concentrations varying between 1 pM and 1 μM. However, if the cultured cells were deprived from serum for 48 h, or when they were pre-incubated with the glucocorticoid receptor-blocking agent RU 38486, CRH-stimulated ACTH release was already significantly suppressed by 1 pM, and maximally suppressed by 0.1 nM SRIF. Preincubation with 5 nM dexamethasone (DEX) completely abolished the sensitivity of ACTH release to SRIF (103). These early *in vitro* studies suggested that normal corticotrophs only respond to SRIF with inhibition of ACTH release if the cells have been cultured in glucocorticoid-free medium, *i.e.* sensitivity to SRIF-induced inhibition of ACTH by the anterior pituitary is only observed when the physiological feedback regulation of ACTH release by glucocorticoids fails (104). In agreement with this hypothesis, ACTH secretion in normal individuals is not affected by infusions of natural SRIF and OCT (105, 106), but in patients with Addison's disease SRIF suppresses ACTH levels (107). The influence of the microenvironment in which the anterior pituitary cells have been cultured, to alter somatotroph and lactotroph sensitivity for SRIF as well, underlines its physiological relevance. Preincubation of rat anterior pituitary cells with either low (5 nM) or high (100 nM) concentrations of DEX significantly decreased the sensitivity of GH release to SRIF (108). Also, estrogens have been demonstrated by several groups to induce the inhibitory effects of SRIF on prolactin (PRL) release by lactotrophs, most likely caused by an increase in the number of sst sites on normal lactotrophs after estrogen pre-treatment (109, 110).

SRIF in corticotroph adenomas

As already briefly discussed, various anecdotal reports concerning the current clinically available sst_2-preferring SRIF-analog OCT, do not demonstrate clinical efficacy in treating Cushing's disease (Table II). Regulation of the expression of sst on corticotroph adenomas by glu-

cocorticoids, may form a plausible explanation, *i.e.* the high levels of cortisol in patients with pituitary dependent Cushing's disease can thus be responsible for the observed lack of inhibition of ACTH release by SRIF and/or OCT (93-95). Moreover, in patients with Nelson syndrome and adrenal insufficiency of different origin, SRIF and/or OCT lower ACTH secretion (91, 93, 107, 111-114) (Table II). These observations are in agreement with various reports demonstrating that in eight ACTH-secreting pituitary adenomas (patients with untreated Cushing's disease) no increased uptake of ^{111}In-diethylenetriamine pentaacetic acid (DTPA) octreotide, an sst_2-preferring radiolabeled SRIF-analog, was found (115, 116), whereas ^{111}In-DTPA scintigraphy is positive in two invasive ACTH-secreting macroadenomas and two cases of Nelson's syndrome (115, 117). In agreement with SRIF and OCT-mediated inhibition of ACTH release in normal corticotroph cells, ACTH release from corticotrophinomas seems only sensitive to OCT in the absence of peripheral feedback regulation by glucocorticoids, suggesting that the sst_2 might be down-regulated when cortisol levels are high (103, 104).

SRIF receptor modulation, *i.e.* down-regulation, by glucocorticoids was already demonstrated in the early 1980s. Chronic glucocorticoid treatment of GH_4C_1 cells, a clonal strain of rat pituitary tumor cells, and AtT20/D16 cells, a clonal strain of mouse corticotroph tumor cells, caused a 20-40% decrease in [^{125}I-Tyr^1]-SRIF binding (118). Furthermore, with respect to SRIF and SRIF-analog mediated inhibitory effects on ACTH release by corticotroph tumor cells, numerous reports have been published in which consensus is found that SRIF, as well as sst_2- and sst_5-preferring SRIF analogs, lower ACTH release in mouse corticotroph tumor cells (119-124). The consequences of SRIF receptor down-regulation by glucocorticoids on the biological responsiveness of corticotroph adenoma cells was demonstrated in primary cultures of human corticotroph adenomas, in which OCT-induced inhibition of basal and CRH-induced ACTH release, was abolished if the cells were pretreated with hydrocortisone (94). Altogether, the lack of suppressive action of sst_2-preferring SRIF-analogs in patients with untreated Cushing's disease seems to be prohibited due

to the hypercortisolemic condition of the patients, which is suggested to down-regulate sst_2 expression of the corticotroph adenoma. However, little is known with respect to the quantitative expression of the five sst subtypes in tumoral corticotroph cells. Several studies reported the highest frequency of expression of sst mRNA for sst_2 (88%) and sst_5 (86%), and to a lesser extent sst_1 (63%) (125-128). Although sst_2 mRNA are detectable in corticotroph adenomas, their levels of expression are apparently low, since OCT has no effect ACTH secretion in pituitary dependent Cushing's disease.

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Chapter II

Aim of the Thesis

The main goal of this thesis is to evaluate novel patho-physiological aspects of the functional role of human somatostatin receptor subtypes (sst) in pituitary adenomas, in particular the role of sst_2 and sst_5 in the regulation of hormonal hypersecretion by GH/PRL and ACTH-secreting pituitary adenomas.

In general, although the clinical introduction of sst_2-preferring SRIF-analogs has nowadays emerged into their use as reference drug for the medical treatment of acromegaly, still, no more than approximately two thirds of patients with persistent acromegaly respond satisfactory to these agents. The novel multiligand SOM230, investigated in several animal models as well as in phase I studies, can become a novel medical tool in the treatment of acromegaly. This brings us to the following questions:

Is SOM230 capable of lowering hormonal hypersecretion from human GH-secreting pituitary adenomas, both *in vitro* and *in vivo*?

Which sst subtypes are involved in SOM230- and OCT-mediated GH inhibition?

Is SOM230 a safe drug for acromegalic patients?

First, the *in vitro* efficacy of SOM230 compared with OCT will be carried out in a series of primary GH-secreting pituitary adenoma cell cultures. Quantitative RT-PCR shall be performed to analyse sst mRNA expression levels of the adenomas which, in addition, will be correlated to the inhibitory effects of SOM230 and OCT on GH release by the adenoma cells. Second, we will perform the first proof-of-concept trial in acromegaly to assess the efficacy of SOM230, compared with OCT, on circulating GH, total & free IGF-I concentrations. However, even though a mild transient hyperglycaemia is observed as an adverse effect in acromegalic patients treated with OCT or Lanreotide, due to the lowering of circulating insulin levels, it is not known whether SRIF-analogs can modulate insulin signaling as well. Therefore, circulating insulin

levels as well as plasma glucose and IGFBP-1 levels will be assessed, to evaluate whether SOM230 alters glucose homeostasis differently as compared with OCT. Additional analyses to evaluate quantitative sst subtype mRNA expression in visceral adipose fat tissue, a peripheral target tissue of insulin action, will be carried out. Also, with the use of a human hepatoma cell line, as the liver forms another peripheral target tissue of insulin action, we will evaluate whether SRIF-analogs can influence insulin-responsiveness directly at the level of the insulin receptor.

Several reports have evaluated the inhibitory efficacy of sst_2 and sst_5 activation in the regulation of GH secretion by use of primary cultures of GH-secreting pituitary adenomas. It has been suggested that the sst_2 seems the (pre-) dominant receptor in regulating GH release by GH-secreting pituitary adenoma cells, whereas sst_5 receptors may mediate an inhibitory effect on GH secretion as well. A direct proof for this hypothesis is currently lacking, however, which leads to the following question:

Based on a fundamental approach, is there support for a functional interplay between sst_2 and sst_5?

No report yet investigated in a basic experimental design, whether these two sst subtypes have a functional interplay with each other. Therefore, we will prosecute the pharmacological profiling of several SRIF-analogs as determined by inhibition of cAMP release and radioligand SRIF-14 membrane binding in HEK 293 cells expressing different sst_2/sst_5 ratios. Also, over the past years RNA interference has exploded onto the research scene as a new approach to manipulate gene expression in mammalian systems. On the basis of a new mammalian expression vector that directs the synthesis of short hairpin siRNA-like transcripts, we will synthesize a siRNA construct for sst_5 and evaluate its efficacy to down-regulate sst gene expression in transfected mammalian cell lines. Although SRIF analogs, as neuro-modulatory agents, have been proposed as a medical treatment option for patients with Cushing's dis-

ease, no efficacy in terms of lowering ACTH or cortisol levels have been reported with sst_2-preferring SRIF-analogs. This is presumably due to a glucocorticoid-induced down regulation of sst_2. However, not much is known yet about the role of SRIF-analogs targeting sst_5 receptors in the regulation of ACTH secretion, and in addition, quantitative data concerning sst expression levels in ACTH-secreting pituitary adenomas are lacking. This raises several questions:

Which is the quantitative sst subtype expression pattern is observed in human ACTH-secreting pituitary adenomas?

Do SRIF-analogs, targeting sst_5 or sst_2 and sst_5 mediate ACTH release differently as compared with sst_2-preferring SRIF-analogs?

In a series of ACTH-secreting pituitary adenoma cell cultures, obtained after transsphenoidal surgery of patients diagnosed with Cushing's disease, quantitative RT-PCR will elucidate sst mRNA expression in these adenomas. We will also compare the *in vitro* inhibitory effects of SOM230 and OCT on basal and corticotropin-induced ACTH release from primary human ACTH-secreting adenoma cells, as well as from mouse ACTH-producing corticotroph tumor cells. Functional properties of sst_2 and sst_5 receptors, endogenously expressed in mouse ACTH-secreting corticotrophs, will be investigated in more detail to further explore the patho-physiological role of both sst subtypes, especially in the presence of glucocorticoids, in the regulation of ACTH release.

Regarding the proposed role of novel SRIF analogs in the treatment of neuroendocrine tumors, novel subtype specific and universal SRIF analogs shall be critically evaluated with particular emphasis on their clinical potential and pitfalls, such as tachyphylaxis to OCT and Lanreotide treatment in a subgroup of neuroendocrine tumors. Several questions do remain unanswered:

Is tachyphylaxis sst subtype specific?

Why do chimeric SRIF-DA agonists display enhanced efficacy compared with the combined treatment of the individual SRIF and DA compounds?

To address the topic of tachyphylaxis, a series of sst subtype specific SRIF-analogs, as well as the universal ligand SOM230 will be used in transiently transfected sst_2 and/or sst_5-expressing HEK293 cells, to analyse functional responses, in terms of adenylyl cyclase inhibition, after prolonged pre-treatment in vitro with the same panel of SRIF-analogs. Functional characterisation of BIM-23A760, representing a group of chimeric compounds that have been launched recently being one of the potential novel medical tools for the treatment of neuroendocrine tumors, will be performed. BIM23A760, targeting both sst_{2+5} and dopamine D2 (D2R) receptors will be tested in primary GH-secreting pituitary adenoma cell cultures as well as in a series of sst_2+sst_5+D2R transfected mammalian cells, to retrieve additional functional insights with respect to the potency of these chimeric molecules.

Finally, in the general discussion the results of the studies reported in this thesis are evaluated, and we hypothesize on the possible clinical significance and potential future developments.

Chapter III

Clinical Potential of SOM230 in the Medical Treatment of Acromegaly

Chapter III-1

The novel somatostatin analog SOM230 is a potent inhibitor of hormone release by growth hormone and prolactin secreting pituitary adenomas in vitro

J Clin Endocrinol Metab. 2004, 89 (4): 1577-1585

Abstract

To determine the inhibitory profile of the novel SRIF-analog SOM230 with broad SRIF receptor binding, we compared the in vitro effects of SOM230, octreotide (OCT), and SRIF-14 on hormone release by cultures of different types of secreting pituitary adenomas. OCT (10nM) significantly inhibited GH release in 7 of 9 GH-secreting pituitary adenoma cultures (range -26 to -73%), SOM230 (10nM) in 8/9 cultures (range -22 to -68%), and SRIF-14 (10nM) in 6/6 cultures (range -30 to -75%). Sst analysis showed predominant, but variable levels of sst_2 and sst_5 mRNA expression. In one culture, completely resistant to OCT, SOM230 and SRIF-14 significantly inhibited GH release in a dose-dependent manner with an IC_{50} value in the low-nanomolar range. In the other cultures SOM230 showed a lower potency of GH release inhibition (IC_{50} 0.5 nM), compared to OCT (IC_{50} 0.02 nM) and SRIF-14 (IC_{50} 0.02 nM). A positive correlation was found between sst_2-, but not sst_5 mRNA levels, in the adenoma cells and the inhibitory potency of OCT on GH release *in vivo* and *in vitro*, and the effects of SOM230 and SRIF-14 *in vitro*. In 3 prolactinoma cultures, 10nM OCT weakly inhibited PRL release in only one (-28%), whereas 10nM SOM230 significantly inhibited PRL release in 3/3 cultures (-23, -51 and -64.0%). The inhibition of PRL release by SOM230 was related to the expression level of sst_5, but not sst_2 mRNA. Conclusions: 1) SOM230 has a broad profile of inhibition of tumoral pituitary hormone release in the low nanomolar range, probably mediated via both sst_2 and sst_5 receptors. The higher number of responders of GH-secreting pituitary adenoma cultures to SOM230, compared to OCT, suggest that SOM230 has the potency to increase the number of acromegalic patients which can be biochemically controlled. 2) Compared to OCT, SOM230 is more potent in inhibiting PRL release by mixed GH/PRL secreting adenoma- and prolactinoma cells.

Introduction

Current treatment options in patients with acromegaly due to a growth hormone (GH) secreting pituitary adenoma are surgery, medical therapy and radiotherapy. As medical therapy, stable somatostatin (SRIF) analogs, such as octreotide (OCT) and the sustained release depot formulations Sandostatin-LAR and SR-lanreotide are widely used, both as primary or secondary therapy (1). Treatment with this generation of oc-

tapeptide SRIF-analogs results in clinical and biochemical control, i.e. normalization of circulating GH and IGF-I levels, in approximately two-thirds of the acromegalic patients (1). The successful medical treatment of acromegaly with octapeptide SRIF-analogs is due to the expression of high affinity (density) SRIF receptors on the adenoma cells, mainly sst_2, which is one of the five known SRIF receptor subtypes, sst_{1-5}, and the selective high affinity binding of these analogs to the sst_2 subtype (2). The molecular basis for the clinical experience that one-third of patients with acromegaly are not adequately controlled by treatment with octapeptide SRIF-analogs is probably formed by a variable expression of sst_{1-5} in the adenomas of these patients (3-9). Several groups have demonstrated that sst_2 and sst_5 receptors are the most important sst involved in the regulation of GH secretion (2, 9). Using sst selective SRIF-analogs it was shown that, in adenomas which were poorly responsive to the sst_2 selective agonists OCT and lanreotide, sst_5 selective or sst_2-sst_5 bi-specific compounds were able to suppress GH release more potently (10, 11). These observations initiated the development of novel stable SRIF-analogs with a more universal sst binding profile. One of these new compounds, SOM230, was recently shown to reduce circulating IGF-I levels in rats by 75% after 126 days of continuous infusion. This effect was significantly more potent compared to OCT, which suppressed IGF-I release under the same experimental condition by only 28% (12). In rats, the terminal elimination half-life was 23h, which is approximately 11 times longer compared to OCT (12). Finally, both in cynomolgus monkeys and beagle dogs, infusion of SOM230, and to a much lesser extent OCT (SMS 201-995), lowered IGF-I levels potently (13). Compared with OCT, SOM230 has a 30-, 5- and 40-times higher binding affinity to sst_1, sst_3 and sst_5 receptors, respectively, and 2.5 times lower affinity to sst_2 (12). This universal binding profile of SOM230 to sst (Table I), in combination with the importance of sst_2 and sst_5 receptors in regulating GH secretion by pituitary adenoma cells, was the rationale for the present study to evaluate, for the first time, the effects of SOM230, OCT and the native SRIF molecule SRIF-14 on hormone secretion by human GH-secreting pituitary adenomas. Moreover, recent evidence suggests that sst_5 receptors are also

involved in the regulation of PRL secretion by human prolactinoma cells (11, 14). Therefore, we compared the effects of OCT and SOM230 on PRL secretion by primary cultures of human prolactinomas. A correlation is made with the sst subtype expression pattens in the adnoma cells.

Table I. Binding affinity of SRIF-14, OCT and SOM230 for sst_{1-5}.

Compound	$hsst_1$	$hsst_2$	$hsst_3$	$hsst_4$	$hsst_5$
SRIF-14	0.93 ± 0.12	0.15 ± 0.02	0.56 ± 0.17	1.5 ± 0.4	0.29 ± 0.04
OCT	280 ± 80	0.38 ± 0.08	7.1 ± 1.4	>1000	6.3 ± 1.0
SOM230	9.3 ± 0.1	1.0 ± 0.1	1.5 ± 0.3	>100	0.16 ± 0.01

Data are reproduced with permission (12) Results are the mean ± SE, IC50 values are expressed in nM.

Materials and Methods

Patients

Pituitary tumor samples were obtained by transsphenoidal operation from 9 patients with GH-secreting pituitary adenomas and from 3 patients with prolactinomas as described in detail previously (15). Diagnosis was made on the basis of clinical and biochemical characteristics of the patients, in combination with (immuno)histochemistry of the tumor samples. All patients gave their informed consent for the use of tumor material for research purposes. Directly after obtaining the tissue, a piece of tissue was snap-frozen on dry ice and stored at -80 C until analysis. The remaining tissue was used for cell culture. To evaluate in vivo responsiveness to OCT, patients received at 9.00 a.m. a single subcutaneous (s.c.) injection of 100μg OCT. Blood samples were collected 30 minutes before and 1 minute before, and hourly until 8 hours after s.c. injection. At the same time points, blood samples were taken at a control day. The effect of OCT on circulating GH levels was determined by calculating the mean GH suppression between 2-8 h after s.c. injection, compared to the same period of time on a control day.

Quantitative PCR

Quantitative PCR was performed as described previously (16). Briefly, poly A^+ mRNA was isolated using Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway) from freshly isolated pituitary adenoma cell pellets containing 0.5-1.0x10^6 adenoma cells. Analysis of sst subtype mRNA levels in both tissue or freshly isolated cell pellets (n=4) yielded comparable results (data not shown). cDNA was synthesized using the poly A^+ mRNA, which was eluted from the beads in 40 µl H_2O for 10 minutes at 65 °C, using Oligo $(dT)_{12-18}$ Primer (Invitrogen, Breda, The Netherlands). One-twentieth of the cDNA library was used for quantification of sst subtype mRNA levels. A quantitative PCR was performed by TaqMan® Gold nuclease assay (Perkin Elmer Corporation, Foster City, CA, USA) and the ABI PRISM® 7700 sequence Detection System (Perkin Elmer) for real-time amplifications, according to manufacturer's protocol. The assay was performed using 15µl TaqMan® Universal PCR Master Mix (Applied Biosystems, The Netherlands) , 500 nM forward primer , 500 nM reverse primer , 100 nM probe and 10µl cDNA template, in a total reaction volume of 25 µl. The reactions were carried out in a ABI 7700 Sequence Detector (Perkin Elmer, The Netherlands). After an initial heating at 95° C for 8 minutes, samples were subjected to 40 cycles of denaturation at 95° C for 15 seconds and annealing for 1 minute at 60° C. The primer and probe sequences that were used are indicated below. The detection of hypoxantine-phosphoribosyl-transferase (hprt) mRNA served as a control and was used for normalisation of the sst subtype mRNA levels. The primer sequences that were used included:

hprt forward 5'-TGCTTTCCTTGGTCAGGCAGTAT-3'
hprt reverse 5'-TCAAATCCAACAAAGTCTGGCTTATATC-3'
sst_1 forward 5'-TGAGTCAGCTGTCGGTCATC-3'
sst_1 reverse 5'-ACACTGTAGGCACGGCTCTT-3'
sst_2 forward 5'-TCGGCCAAGTGGAGGAGAC-3'
sst_2 reverse 5'-AGAGACTCCCCACACAGCCA-3'
sst_3 forward 5'-CTGGGTAACTCGCTGGTCATCTA-3'
sst_3 reverse 5'-AGCGCCAGGTTGAGGATGTA-3'

sst_5 forward 5'-CATCCTCTCCTACGCCAACAG-3'
sst_5 reverse 5'-GGAAGCTCTGGCGGAAGTT-3'

The probe sequences that were used included:
hprt 5'-FAM-CAAGCTTGCGACCTTGACCATCTTTGGA-TAMRA-3'
sst_1 5'-FAM-ACAGCTGCGCCAACCCCATC-TAMRA-3'
sst_2 5'-FAM-CCGGACGGCCAAGATGATCACC-TAMRA-3'
sst_3 5'-FAM-CGGCCAGCCCTTCAGTCACCAAC-TAMRA-3'
sst_5 5'-FAM-CCCGTCCTCTACGGCTTCCTCTCTGA-TAMRA-3'

Primers and probes were purchased from Biosource (Nivelles, Belgium). The relative amount of sst subtype mRNA was determined using a standard curve generated from known amounts of human genomic DNA. For determination of the amount of hprt mRNA, a standard curve was generated of a pool of cDNAs from a human cell line known to express hprt. The linear range of amplification ranged between 4 log dilutions of genomic DNA or cDNA, respectively. The relative amount of sst subtype mRNA was calculated relative to the amount of hprt mRNA and is given in arbitrary units. Each sample was assayed in duplicate.

Cell dispersion and cell culture

Single cell suspensions of the pituitary adenoma tissues were prepared by enzymatic dissociation with dispase as described in detail previously (15). For short-term incubation of monolayer cultures, the dissociated cells were plated in 48-well plates (Corning, Cambridge, MA) at a density of 10^5 cells per well per 1 ml culture medium. After 3-4 days the medium was changed and 72-h incubations without or with test-substances were initiated. At the end of the incubation the medium was removed and centrifuged for 5 min. at 600xg. The supernatant was collected and stored at -20^{o}C until analysis. The choice for a 72h incubation was made on the basis of previous studies, in which we demonstrated that exposure of GH-secreting pituitary adenoma cells for 4-96 h to octreotide

showed a variable, but in all instances during longer incubations statistically significant inhibition of GH release, which paralleled the sensitivity of GH secretion to octreotide in vivo (17). For long-term incubation studies in Transwells® (18), the isolated tumor cells were plated in Transwell-COL® membranes (Corning) at a density of 10^5 cells per well. The Transwells® were then placed into multiwell plates (24-well, Corning) containing 1 ml culture medium. After 72 hr the Transwells® were transferred to wells containing fresh medium (without or with test-substances). Every 3-4 days the cells were placed into fresh medium and the incubation media were collected and stored at -20°C until determination of hormone concentrations. The cells were cultured at 37 C in a CO_2-incubator. The culture medium consisted of Minimum Essential Medium with Earle's salts (MEM) supplemented with non essential amino acids, sodium pyruvate (1 mmol/L), 10% fetal calf serum (FCS), penicillin ($1x10^5$ U/L), fungizone (0.5 mg/L), L-glutamine (2mmol/L), and sodium bicarbonate (2.2 g/L), pH 7.6. Media and supplements were obtained from GIBCO Bio-cult Europe (Invitrogen, Breda, The Netherlands). Unfortunately, generally not enough tumor material was obtained to test for each tumor the dose-dependency of effects for the indicated drugs.

Hormone determinations

Human GH and PRL concentrations in the media and cell extracts were determined by a non-isotopic, automatic chemiluminescence immunoassay system (Immulite, DPC Inc., Los Angeles, CA). Intra- and interassay CV's for GH and PRL were 6.0%, 5.7% and 6.2%, 6.4%, respectively. ACTH, LH and FSH concentrations in the culture media were determined as well, in order to exclude the presence of contaminating normal pituitary cells in the cultures. Human ACTH, LH and FSH concentrations were determined by by a non-isotopic, automatic chemiluminescence immunoassay system (Immulite, DPC Inc.). Intra- and interassay CV's for ACTH, LH and FSH were 5.6%, 5.7%, 6.4% and 7.8%, 12.3%, 7.5%, respectively. Except for the expected hormones GH and PRL, none of the other hormones were detectable (not shown).

Test-substances

OCT (Sandostatin®) and bromocriptine were obtained from Novartis Pharma A.G. (Basle, Switzerland). SOM230 was provided by Novartis Pharma A.G. Somatostatin-14 (SRIF-14) was purchased from Sigma Chemical Co. (St. Louis, MO).

Statistical analysis of the data

All data on hormone release are expressed in mean ± SE, n=4 wells per treatment group. All data were analyzed by analysis of variance (ANOVA) to determine overall differences between treatment groups. When significant differences were found by ANOVA, a multiple comparison between treatment groups was made using the Newman-Keuls test. Correlation analysis was done by the use of the Spearman's rank correlation test. Calculation of IC_{50} values for inhibition of hormone release were made using GraphPad Prism (San Diego, CA).

Results

Sst subtype mRNA expression and correlation with in vivo GH suppression by octreotide

In 7 out 9 GH-secreting pituitary adenoma samples the sst subtype mRNA expression pattern was determined. Figure 1 shows that 2 adenomas expressed sst_1 mRNA, three adenomas expressed sst_3 mRNA, and that all adenomas expressed sst_2 and sst_5 mRNAs. Expression of sst mRNAs was variable between adenoma samples. The difference between the lowest and the highest level measured, amounted to 7-, 8-, 3- and 5-fold for sst_1, sst_2, sst_3 and sst_5, respectively. The sst subtype mRNA levels showed no statistical correlation (data not shown). The mean percentage *in vivo* GH suppression (range +18 and -91% suppression) between 2-8 hr following the administration of a single s.c. dose of 100 µg OCT (patient no. 1, 2, 3, 5, 6 and 7) was positively correlated with sst_2 mRNA expression levels (fig. 2, left panel), but not with sst_5 mRNA levels (fig. 2, right panel).

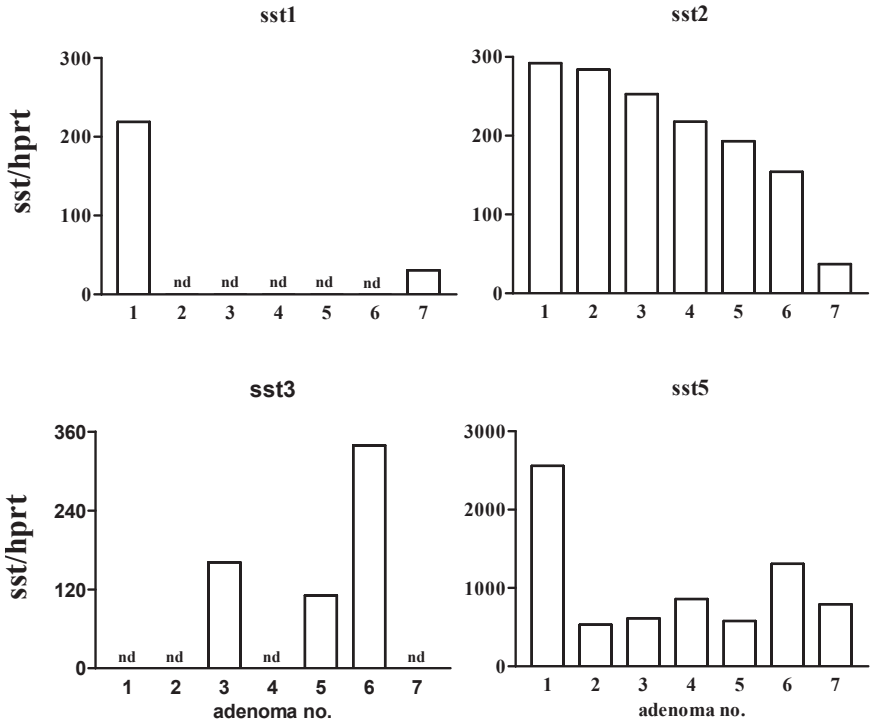

Figure 1. Relative sst_1, sst_2, sst_3, and sst_5 mRNA expression levels in GH-secreting pituitary adenomas. Values are expressed as the number of copies of the respective sst mRNA's, relative to the number of copies of hprt. nd= not detectable. Note the differences in the scale of the y-axis between sst_1, sst_2 and sst_3 mRNA levels on the one hand, and the higher sst_5 mRNA levels on the other hand. The relative amount of sst subtype mRNA was calculated relative to the amount of hprt mRNA and is given in arbitrary units.

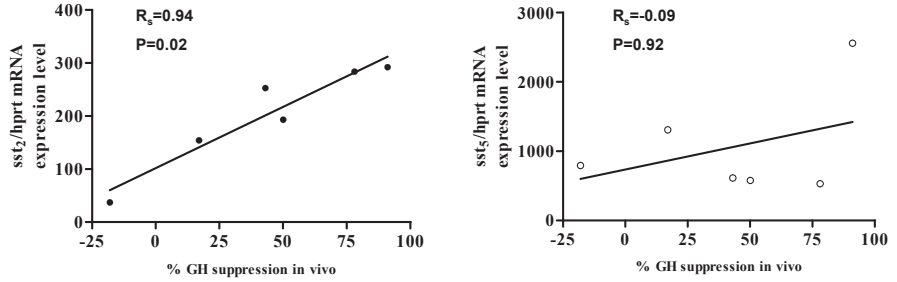

Figure 2. Correlation between sst_2 and sst_5 mRNA levels and the in vivo response of GH release to a single s.c. injection of 100µg of OCT in six acromegalic patients. The relative amount of sst subtype mRNA was calculated relative to the amount of hprt mRNA and is given in arbitrary units.

Effects of SRIF-analogs on GH secretion

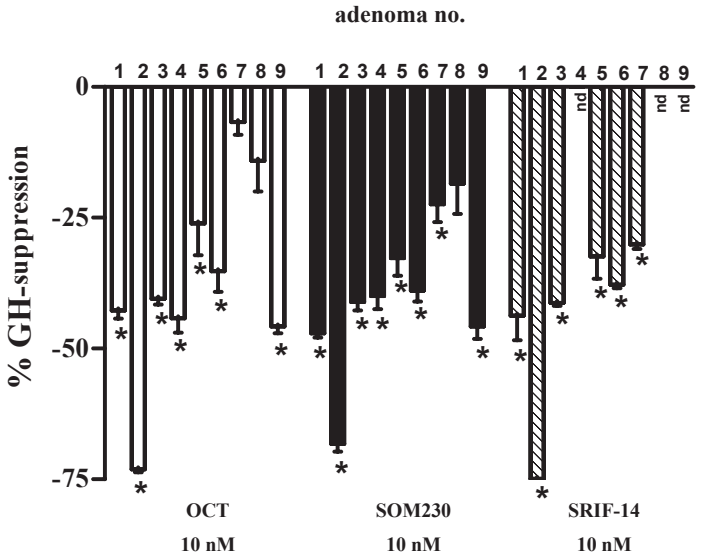

Figure 3. Effects of OCT (10nM; open bars), SOM230 (10nM; filled bars) and SRIF-14 (10nM; hatched bars) on GH release by 9 cultured GH-secreting pituitary adenomas. The results are expressed as the percentage inhibition of GH release compared to untreated, control cells. *$p<0.01$ vs untreated control cells. nd= not done.

In most GH-secreting pituitary adenomas the effect of 10nM OCT, SOM230 or SRIF-14 on GH release was evaluated in parallel in vitro. Figure 3 shows that GH release was significantly suppressed by OCT in 7 out of 9 cultures, by SOM230 in 8 out of 9 cultures and by SRIF-14 in 6 out of 6 cultures. In the majority of the adenomas, the percentage suppression using a maximally active concentration of the three compounds, was comparable. Only the adenoma cells of patient 7 showed a dissociated response to OCT on the one hand and to SOM230 and SRIF-14 on the other hand. In this adenoma culture, GH release was not inhibited by OCT, whereas both SOM230 and SRIF-14 induced a statistically significant suppression of GH release. In the respective patient, OCT 100 µg s.c. did not suppress GH concentrations as well. As seen in figure 3, the response in terms of GH-suppression was variable between the individual adenoma cultures. This variable responsiveness to OCT and SOM230 correlated well with the sst_2 mRNA expression levels in

the adenoma cells of the respective patients (fig. 4, upper panel). Comparable to the patients responsiveness to OCT, sst_5 mRNA expression showed no statistically significant correlation with the percentage of GH suppression by 10nM OCT. Interestingly, the effects of 10nM SOM230, which shows a more universal binding profile for sst receptors, including high affinity binding to sst_5, also showed no correlation with sst_5 mRNA expression levels. This is demonstrated in figure 4, lower panel.

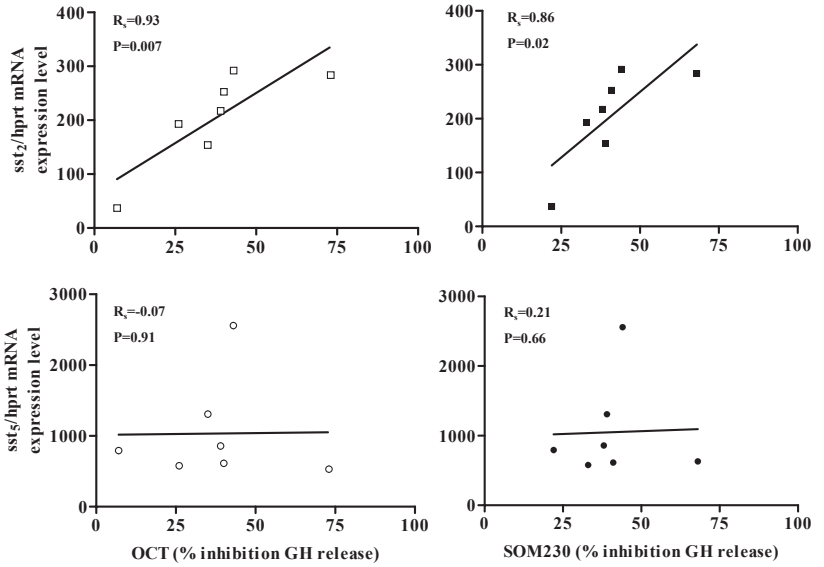

Figure 4. Correlation between sst_2 (upper panel) and sst_5 (lower panel) mRNA levels and the percentage suppression of GH release induced by SOM230 (10nM) and OCT (10nM). The relative amount of sst subtype mRNA was calculated relative to the amount of hprt mRNA and is given in arbitrary units.

In several adenoma cultures, the dose-response relationship of the inhibitory effects of OCT, SOM230 and SRIF-14 could be evaluated. Figure 5, right panel shows the effects of the three compounds on GH release by the adenoma cells of patient 7, which did not respond to OCT. The adenoma cells of this patient had the lowest sst_2 mRNA levels of all cases studied (fig. 1, upper right panel). As shown, GH release by the cells of this patient was inhibited in a dose-dependent manner by both SOM230 and SRIF-14. The IC_{50} values for the inhibition of GH release were 0.5 and 0.6 nM for SOM230 and SRIF-14, respective-

ly. In the other adenoma cultures both OCT and SRIF-14 were slightly more efficacious, in terms of IC_{50} values, compared with SOM230. The left panel of figure 5 shows the mean dose-response of OCT, SOM230 and SRIF-14 for the other cultures. Mean IC_{50} values were 0.02, 0.5 and 0.02 nM for OCT, SOM230 and SRIF-14, respectively.

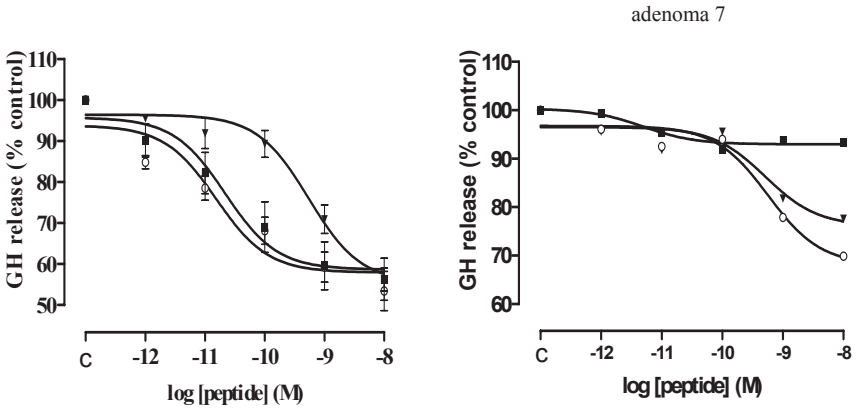

Figure 5. Dose-dependent inhibition of GH release by SOM230 ▼, OCT ■ and SRIF-14 ○ in cultured pituitary adenoma cells. The left panel represents the mean dose-dependent effects of SOM230 (n=5; adenoma no. 2, 3, 4, 5 and 6), OCT (n=4; adenoma no. 2, 3, 5 and 6) and SRIF-14 (n=4; adenoma no. 2, 3, 5 and 6). The right panel shows the dose-dependent inhibitory effects of the compounds on GH release by cells patient 7.

Effects of SRIF-analogs on PRL secretion

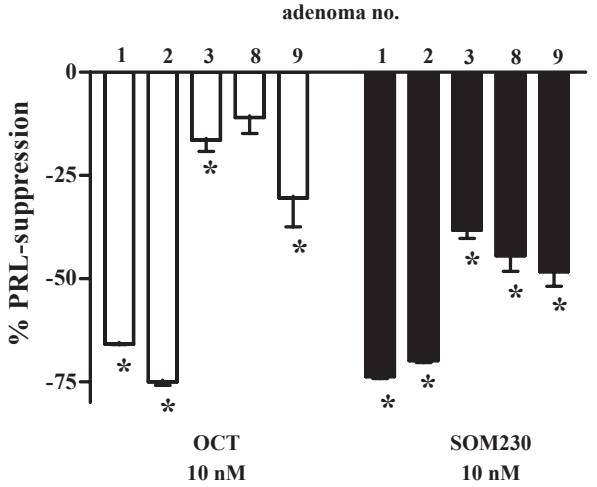

Figure 6. Effect of 10nM OCT (open bars) or 10nM SOM230 (filled bars) on PRL secretion by mixed GH/PRL secreting pituitary adenomas. The results are expressed as the percentage inhibition of PRL release compared to untreated, control cells. *p<0.01 vs untreated control cells.

Because previous studies demonstrated the involvement of sst_5 receptors in regulating PRL release, we also compared the effects of OCT and SOM230 on PRL release. Five adenoma cultures co-secreted PRL as well. OCT (10nM) and SOM230 (10nM) significantly inhibited PRL release in 4/5 and 5/5 cultures, respectively (fig. 6). The percentage

Table II. Patient and hormone data of the 12 patients with pituitary adenoma

Patient no.	Sex	Age	GH	PRL	IGF-I	In vitro hormone release	
GH-secreting adenoma	M/F	Yr	µg/L	µg/L	nmol/L	GH ($ng/10^5$ cells/72h)	PRL ($ng/10^5$ cells/72h)
1	M	58	9.8	19	175	520±24	1192±60
2	M	26	31	111	201	547±7	492±7
3	F	60	5.4	39	66	372±13	1105±71
4	F	55	15	19	184	1021±19	-
5	M	36	118	24	111	336±35	-
6	F	44	14	15	289	230±10	-
7	F	41	72	6.7	285	640±18	-
8	F	42	6.8	0.9	88.3	55±2	21±2
9	F	65	6.8	9.3	169	246±9	156±5
Prolactinoma							
10	F	35		2000		-	5638±143
11	F	64		13520		-	1486±80
12	F	37		32		-	1700±50

Normal range for IGF-I: 12-40 nmol/L.
- means not detectable

suppression was between 16 and 66% for OCT and between 38 and 74%, for SOM230, indicating its higher efficacy. In 4/5 cultures (no. 1, 3, 8 and 9), SOM230 was significantly more potent with regards to its maximal suppressive effect on PRL release, when compared to OCT ($p<0.01$). In addition to mixed GH/PRL secreting pituitary adenomas, we also compared the effects of OCT and SOM230 on PRL release in 3 primary human prolactinoma cultures (no. 10, 11 and 12) (table II). Figure 7 shows

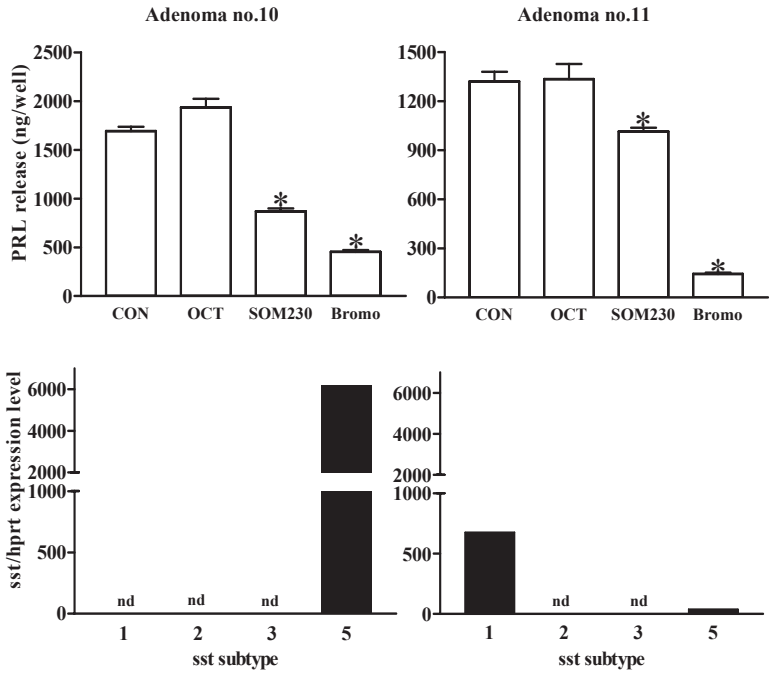

Figure 7. Upper panel: Effects of SOM230 (10nM), OCT (10nM) and bromocriptine (10nM) on PRL release by 2 cultured prolactinomas (no. 10 and 11). *p<0.01 vs untreated control cells. Incubation time: 72 hr. Lower panel: sst subtype mRNA expression in the adenoma cells of patient 10 and 11.

that prolactinoma no.10 selectively expresses a high level of sst_5 mRNA, whereas prolactinoma 11 expressed sst_1 mRNA but very low sst_5 mRNA. In agreement with the low affinity of OCT for sst_5 and the very high affinity of SOM230 for this sst, 10nM OCT did not significantly inhibit PRL release by the two prolactinoma cultures, whereas 10nM SOM230 potently suppressed (-49%) PRL release by cells of adenoma 10 (high sst_5), and slightly (-23%) by cells of prolactinoma no.11 (low sst_5). Both adenoma cultures showed high sensitivity to 10nM of the dopamine agonist bromocriptine (-73 and -89% inhibition in cultures 10 and 11, respectively). In one prolactinoma culture (no. 12) the long-term in vitro effects of OCT, SOM230 and bromocriptine were studied. Figure 8 shows that both SOM230 (10nM) and bromocriptine (10nM) suppressed PRL release by approximately 90% after 9 days of continuous incubation with the compounds. In this particular adenoma, OCT was only slight-

ly effective (-30% after 9 days). Unfortunately, not enough tissue was obtained from this patient to study the sst subtype expression pattern.

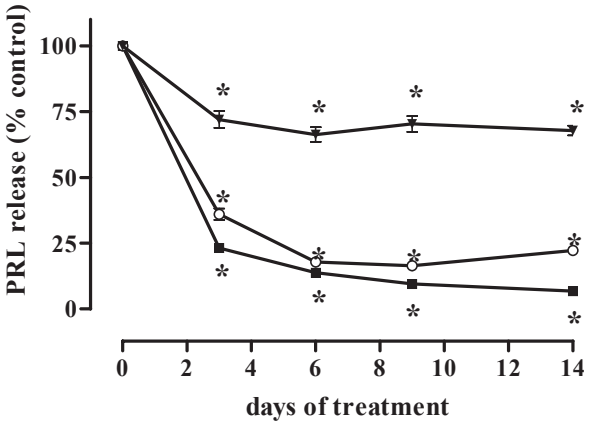

Figure 8. The effect of long-term in vitro incubation with OCT (10nM), SOM230 (10nM) and bromocriptine (10nM) on PRL release by cultured prolactinoma cells of patient no. 12. The pituitary adenoma cells were cultured in Transwell tissue culture inserts for 14 days without or with the compounds indicated. Medium was collected and refreshed every 3 or 4 days and stored at -20 C until determination of PRL concentrations. Values are expressed as the percentage of hormone release of control cells at each time-point indicated and are the mean ± SEM of four wells per treatment group. Symbols represent: ▼OCT, ○ SOM230, ■ bromocriptine. *$p<0.01$ vs untreated control cells. Incubation time: 72 hr.

Discussion

SRIF receptor expression on GH-secreting pituitary adenomas forms the basis for successful treatment of acromegalic patients using sst_2-selective octapeptide SRIF-analogs (19, 20). Recent in vitro studies have demonstrated that both sst_2 and sst_5 receptors are involved in the regulation of GH release by normal human fetal anterior pituitary cells and GH-secreting pituitary adenoma cells (2, 9, 11). In addition, evidence exists that sst_5 exclusively regulates PRL secretion from human prolactinoma cells (11, 14). Moreover, BIM-23244, a SRIF-analog with high affinity binding to sst_2 and sst_5 receptors, was shown to achieve a better suppression of GH secretion by cultured GH-secreting pituitary adenomas which were partially responsive to OCT, suggesting that such bispecific SS-analogs could achieve a better control of GH secretion in a larger number of acromegalic patients (10). The recent observation that sst subtypes may form homo- and heterodimers, resulting in receptors with enhanced bind-

ing affinity and modified functional properties (21, 22), may form one of the explanations for the enhanced efficacy of bi-specific compounds such as BIM-23244. In addition, an sst_2 antagonist was shown to inhibit the GH suppressing action of sst_2/sst_5 bi-selective agonists in human fetal pituitary cells, supporting the concept of functional interaction between sst_2 and sst_5 receptors in the regulation of GH secretion (23). Recently, a novel stable SRIF-analog, with a more universal binding profile and high affinity binding to sst_1, sst_2, sst_3 and sst_5, was introduced (12, 13). This SRIF-analog, SOM230, was shown to be significantly more potent in lowering IGF-I levels in rats, compared with the sst_2-preferring analog OCT (12). SOM230 has a favourable elimination half-life of 23h (12), suggesting that this analog may be suitable for clinical application as well (24). On the basis of the involvement of sst_2/sst_5 receptors in regulating GH secretion and sst_5 receptors in regulating PRL secretion, we compared in the present study the efficacy of SOM230 in the regulation of GH and PRL release by primary cultures of human pituitary adenoma cells. In agreement with previous studies (3-10) we found a variable expression of sst mRNAs, predominantly sst_2 and sst_5, in our series of GH-secreting pituitary adenomas. Only selected cases expressed sst_1 and sst_3 mRNA's, suggesting that these sst subtypes are probably of less importance in this type of pituitary adenoma. Moreover, in most adenomas, sst_5 expression levels were relatively higher compared with sst_2 mRNA levels. On the other hand, the pre-operative response of acromegalic patients to a single s.c. administration of 100μg OCT was positively correlated with the sst_2 mRNA, but not with sst_5 mRNA levels, in the pituitary adenomas of the patients obtained via transsphenoidal surgery. These data demonstrate that the sst_2 subtype is clearly the predominant receptor determining *in vivo* responsiveness to OCT in acromegalic patients. In agreement with previous observations (10), sst_2 mRNA levels were positively correlated with the *in vitro* GH suppression by this sst_2 selective SRIF-analog as well. Surprisingly, although SOM230 shows a very high affinity for sst_5 receptors, no significant correlation was found between the *in vitro* GH suppression by a maximally active concentration of SOM230 and sst_5 mRNA levels, while a positive correlation was found between the effects

of SOM230 and sst_2 mRNA levels. Although these data were unexpected, a previous study by Jaquet et al. (9) in fact already made the same observation using SRIF-14. In this study, sst_2 mRNA, but not sst_5 mRNA levels in GH-secreting pituitary adenomas showed a positive correlation with in vitro GH suppression by 1 nmol/L SRIF-14. As suggested by Jaquet et al. (9), it is not known yet whether the observed sst mRNA levels directly correlate with sst protein levels. The importance of the sst_2 subtype in regulating GH secretion by the majority of human GH-secreting pituitary adenomas is also evident by our observation that the potency of OCT in terms of IC_{50} values measured for the inhibition of GH release are slightly higher, compared with SOM230. Nevertheless, SOM230 shows a high efficacy to inhibit GH release, with an IC_{50} value in the low nanomolar range. In 89% of the cultures SOM230 significantly inhibited GH release. In this respect, OCT is slightly less efficacious, with a significant GH-suppressive effect in 7 out of 9 cultures (78%). In one OCT resistant culture, SOM230 inhibited GH release with an IC_{50} value comparable to that of SRIF-14 (IC_{50} 0.5 and 0.6 nM, respectively), confirming the importance of the sst_5 receptor subtype in mediating GH release, when sst_2 levels are low. However, although SOM230 has a slightly lower potency (IC_{50}) for the inhibition of GH release in most cases, compared with OCT, its efficacy is higher compared with OCT in terms of the number of responders. Therefore, in addition to the sst_2/sst_5 bi-specific compound BIM-23244, also SOM230 has the potential to achieve better control of GH hypersecretion in a larger number of acromegalic patients. Moreover, the very favourable elimination half-life of SOM230 (12) makes this compound an interesting candidate for clinical application as well. Apart from regulating GH secretion, sst_5 receptors play a regulatory role in normal and tumoral PRL secretion as well (2, 11, 14). A significant proportion of GH-secreting pituitary adenomas contain GH and PRL expressing cells, either as individual cells expressing GH or PRL or as mammosomatotroph cells expressing GH and PRL in the same cells (25). In agreement with these data, we observed co-secretion of GH and PRL in 56% of the cultures and showed that SOM230 has a potent inhibitory effect on PRL secretion in mixed GH-PRL secreting pituitary adenomas

and prolactinomas. In 4 out of 5 mixed GH-PRL secreting pituitary adenomas, SOM230 was significantly more potent, compared with OCT, in its maximal suppressive effect on PRL secretion. Interestingly, in the adenoma cells of patient 7, in which SOM230, but not OCT, inhibited GH secretion, also PRL secretion was not suppressed by OCT. PRL secretion was suppressed by approximately 50% by SOM230, however. The parallel responses of GH and PRL secretion in this particular case suggest that the adenoma contains mainly somatomammotroph cells, which were shown to express predominantly sst_5 receptors (3). Recently, it was shown that activation of sst_1 by the sst_1-selective agonist BIM-23296 caused a dose-dependent inhibitory effect in the nanomolar range on GH and PRL secretion by GH-secreting pituitary adenomas. In addition to lowering GH and PRL secretion this SRIF-analog induced a decrease in cell viability as well (26). In our series we found that 2/7 adenomas expressed sst_1. In selected cases, therefore, sst_1, may play a regulatory role on GH-secreting pituitary adenoma cell function as well. In three DA-agonist sensitive prolactinomas, SOM230 was significantly more potent than OCT in lowering PRL secretion. In two of the prolactinomas there was a clear relationship between the expression of sst_5 mRNA in the adenoma cells and the percentage inhibition of PRL secretion by SOM230. In one prolactinoma culture, which expressed high levels of sst_5 mRNA and no other sst mRNAs, PRL secretion was reduced to the same extent as that induced by bromocriptine. One other prolactinoma, which showed a significantly lower responsiveness to SOM230, had very low sst_5 mRNA levels. The lower potency of OCT in reducing PRL secretion by prolactinomas seems related to the very low sst_2 levels, as was demonstrated in a series of 10 prolactinomas by Jaquet et al. (14). These data further underline the role of sst_5 in mediating its suppressive effect on PRL secretion. However, the potential clinical importance of these findings should be considered in view of the very high proportion of patients with prolactinomas responding to DA-agonist treatment with a normalization of PRL levels and tumor shrinkage (27, 28). In addition, Jaquet et al. (14) previously showed that the effects of sst_5 selective compounds on prolactinoma cells are superimposable, at higher concen-

tration to those of the dopamine agonists, but not additive, particularly in adenomas resistant to dopaminergic suppression of PRL release. While sst_5 receptors may mediate antiproliferative effects (29), the role of this receptor in the control of pituitary adenoma cell proliferation is unclear. Indirect evidence for a role of sst_5 in the regulation of human GH-secreting pituitary adenoma cell proliferation was obtained from the observation of a germ line mutation (Arg240Trp) in genomic DNA from pituitary adenoma and peripheral blood mononuclear cells of an acromegalic patient resistant to SRIF-analog treatment and a higher proliferation rate of cells overexpressing this mutant sst_5 receptor, compared to cells expressing wild type sst_5 (30). Whether activation of sst_5 receptors expressed on human GH-secreting pituitary adenomas and prolactinomas also mediates an antiproliferative effect in these cell types, remains to be elucidated, however. Since somatostatin analogs inhibit the secretion of insulin, impaired glucose tolerance was observed after the acute administration of octreotide (31). Recently, we observed similar glucose responses to the acute administration of SOM230 in vivo (32). However, SOM230 did not modify insulin secretion, suggesting another mechanism of action. The inhibitory effect of octreotide on insulin secretion is short-lived, and clinically important effects on carbohydrate metabolism during long-term therapy are not observed (33). Although the acute rise in glucose levels after SOM230 injection requires further attention, preclinical studies in cynomolgus monkeys showed that insulin, glucagon and glucose levels remained unchanged after seven days of high-dose infusion with SOM230 (13). In addition, 126 days treatment of rats with pharmacological doses of SOM230 did not modify plasma glucose levels (12). These data suggest that SOM230 is well tolerated in rats and monkeys with regard to glucose homeostasis. In conclusion, the novel universal SRIF-analog SOM230 is a potent inhibitor of GH and PRL secretion in GH-secreting pituitary adenomas. The higher number of cultures responding to SOM230, compared with OCT, suggests that SOM230 has the potential to increase the number of patients controlled biochemically, both via sst_2 and sst_5. In addition, SOM230 is more potent in its inhibitory effect on PRL secretion in mixed GH/PRL secreting pituitary adenomas. In prolactinoma cultures, sst_5

receptors mediate the potent inhibitory effects of SOM230 on PRL secretion. Since the majority of patients with prolactinomas are successfully treated using DA-agonists, there may be a role for SOM230 in the treatment of prolactinoma patients which are intolerant to DA-agonists.

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Chapter III-2

A single dose comparison of the acute effects between the new somatostatin analog SOM230 and octreotide in acromegalic patients

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Abstract

Somatostatin (SRIF) analogs have been the medical therapy of choice for the treatment of acromegaly. Treatment with the SRIF-receptor (sst) subtype 2 predominant analogs octreotide and lanreotide, induces clinical and biochemical "cure" in about 65% of acromegalic patients. Growth hormone (GH) secreting pituitary adenomas, which are not controlled, also express sst_5. We compared the acute effects of octreotide and SOM230, a new SRIF-analog with high affinity for $sst_{1,2,3,5}$, on hormone release in acromegalic patients. In a single dose proof-of-concept study, 100µg octreotide, 100 and 250µg SOM230 were given sc to 12 patients with active acromegaly. 100 and 250µg SOM230 dose-dependently suppressed GH levels from 2-8 hrs after administration [-38 ± 7.7% *vs.* -61 ± 6.7%, respectively (resp.); $P < 0.01$]. A comparable suppression of GH levels by octreotide and 250µg SOM230 was observed in 8 patients (-72 ± 7% *vs.* -65 ± 7%, resp.). In 3 patients, the acute GH-lowering effect of 250µg SOM230 was significantly superior to that of octreotide (-70 ± 2% *vs.* -17 ± 15%, resp.; $P < 0.01$). In one patient, the GH-lowering effect of octreotide was better than that of SOM230. Furthermore, *in vitro* analysis of adenoma tissue from two operated patients showed relatively high sst_5 and low sst_2 mRNA expression levels in one patient only responsive to SOM230 treatment, suggesting a pivotal role for sst_5 in mediating the suppressive effects of SOM230 in this patient. Tolerability for SOM230 was good. Glucose levels were initially slightly elevated after octreotide and SOM230 compared to control day, whereas insulin levels were only significantly suppressed by octreotide. We conclude that SOM230 is an effective GH-lowering drug in acromegalic patients with the potential to increase the number of patients controlled during long-term medical treatment.

Introduction

In the majority of patients, acromegaly is caused by a growth hormone (GH) secreting pituitary adenoma, resulting in high circulating GH and insulin-like growth factor-I (IGF-I) concentrations. First choice of medical treatment are the somatostatin (SRIF) analogs, a safe and effective strategy, mimicking the action of the native peptide SRIF in its inhibitory effect on GH release by the adenoma cells (1). The first clinically

available SRIF-analog octreotide has been shown to be effective as primary or secondary therapy for acromegalic patients (2-4). Several studies have demonstrated that long-term therapy with octreotide or lanreotide, administered either by subcutaneous (s.c.) or as a long-acting depot preparation by intramuscular injection, induced clinical and biochemical "cure" in about 65% of patients (5-9). Still, a significant percentage of GH-secreting pituitary tumors seems relatively resistant to octreotide and lanreotide, and this may be explained in part by a variable tumoral expression or reduced receptor density of the five known SRIF-receptor (sst) subtypes on the adenomas of these patients (10). Functional evidence for the existence of sst subtypes comes from studies using human fetal pituitary cell cultures in which SRIF regulates GH and thyrotropin secretion mainly by sst_2 and sst_5, and Prolactin (PRL) secretion mainly by sst_5 (11). Most GH-secreting pituitary adenomas predominantly express mRNA for sst_2 and sst_5, while sst_1 and sst_3 are moderately expressed and sst_4 not found (12, 13). SRIF binds with high affinity to all five sst subtypes, whereas octreotide and lanreotide display a high, low and moderate affinity to sst_2, sst_{1+4} and sst_{3+5}, respectively. Saveanu and coworkers compared the *in vivo* sensitivity of GH release for octreotide in nine acromegalic patients with the tumor mRNA expression for sst_2 and sst_5 subtypes (14). It was observed that sst_2 mRNA expression was lower and sst_5 mRNA was higher in adenomas that were partially sensitive to octreotide, compared with octreotide sensitive adenomas. In the group of partially octreotide sensitive tumors, both the sst_5-preferential analog BIM23268, but especially the sst_2 and sst_5 bi-specific compound BIM23244, were quite effective in suppressing GH secretion. These data indicate that due to the heterogeneous expression of sst_2 and sst_5 subtypes in GH-secreting adenomas, a bispecific analog, such as BIM-23244 that can activate both receptors, may achieve a better control of GH hypersecretion of GH-producing pituitary tumors than octreotide. Bruns and coworkers synthesised SOM230, a stable SRIF-analog with a more universal binding profile to sst subtypes (15, 16). By using alanine scanning technology, essential functional groups of the SRIF peptide responsible for the high affinity to all five sst subtypes were detected. Incor-

poration of four synthetic amino acids and two essential amino acids of SRIF into a stable cyclohexapeptide template resulted in SOM230, a compound which binds with a high affinity to sst_1, sst_2, sst_3 and sst_5, and with low affinity to sst_4. In rats, dogs and monkeys, SOM230 potently and dose-dependently decreases GH and IGF-I levels. Only minimal desensitization of the suppressing effect of SOM230 on IGF-I levels under physiological conditions was observed, which is in contrast to what has been seen in rodents with the effect of octreotide. Additional characteristics of SOM230 include a favourable terminal elimination half life of 27 hours (hrs) in humans, as well as the preliminary evidence that glucose levels in rats and dogs remain normal during long-term administration of the compound. In this paper, we present the detailed analysis of the first single dose proof-of-concept study with SOM230 in acromegalic patients. A double-blind, randomised, cross-over study was performed to compare the *in vivo* effects of a single dose SOM230 to octreotide on GH release, to assess its safety and its tolerability in 12 patients with active acromegaly.

Materials and Methods

Patients

Twelve patients with active acromegaly were recruited at the Erasmus Medical Center, Rotterdam, the Netherlands. All subjects had biochemically active disease, with a mean serum GH concentration greater than 5 µg/L during a 5-hour profile and elevated circulating IGF-I levels (age- and sex-adjusted). GH concentration failed to suppress below 1 µg/L after a 2-hour 75-g oral glucose tolerance test (oGTT). Table 1 shows the biochemical characteristics of the 12 patients. One insulin-treated patient with type II diabetes did not undergo an oGTT. Seven patients had been treated before (see below). In those patients who have been medically treated previously, a wash-out period after the last dose of medication had to be at least 1 month, 1 week, 4 months and 1 month for dopamine agonists, subcutaneous formulations of octreotide, depot formulations of long-acting somatostatin analogs and growth hormone receptor antagonists, respectively. One patient had been previously treated by surgery,

medical treatment and irradiation. One patient was treated only with surgery. Two patients had only been medically treated and three patients were treated with surgery and medical treatment. Five patients were newly diagnosed. Patients with compression of the optic chiasm causing any visual field defect or those requiring surgical intervention for relief of any sign or symptom possibly associated with tumor compression, were excluded. The study was approved by the local ethical committee of the Erasmus Medical Center and all patients gave written informed consent.

Table I. Patients'characteristics on study entry.

Patient	Sex	Age (yr)	GH (μg/L)[a]	PRL (μg/L)[a]	IGF-I (μg/L)[b]	GH after oGTT (μg/L)[c]
1	F	43	13.6	13.8	1981	10.1
2	M	34	7.4	18.1	1301	8.3
3	F	55	31.6	3.4	1385	29.1
4	M	48	26.5	7.8	1515	ND
5	F	36	31.1	7.2	941	21.2
6	M	35	48.1	19.1	1729	29.0
7	M	38	19.5	20.9	2027	16.9
8	M	33	11.0	10.3	1920	11.0
9	F	79	19.3	0.7	1148	24.6
10	M	67	6.9	17.7	849	5.5
11	F	52	5.8	9.8	773	2.5
12	F	40	57.4	6.7	2180	75.0

ND, not determined.
a mean fasting GH and PRL values, collected in a 5-h time period. normal values: GH < 5 μg/L, PRL < 25 μg/L (men) or < 44 μg/L (women).
b range in healthy population: 107-497 μg/L.
c serum GH level after a 2-hour 75-g oral glucose tolerance test (normal nadir GH < 1 μg/L after 2 hrs).

Treatment protocol

Patients were hospitalized on the control day for 24 hrs for the assessment of baseline efficacy parameters. On study day 1, 8 and 15, each

patient received at 09:00 a.m. a single s.c. injection of octreotide 100μg, SOM230 100μg or SOM230 250μg in a randomized, double-blinded, crossover fashion with a minimum 6 days of washout between drug treatments. All patients received standarized meals, served at 08:30 a.m., 12:30 p.m. and 17:30 p.m. Blood samples, withdrawn through an indwelling venous catheter placed in the forearm, for the assessment of GH and PRL concentrations were collected at 30 minutes, one minute before, and every hour for 24 hrs after drug administration. This procedure was repeated on all study days. Furthermore, blood samples for glucose and insulin assessments were collected 30 minutes, one minute before and every half hour for 2 hrs after lunch. Blood specimens were centrifuged and the plasma was frozen at -20°C until it was assayed. Safety assessments included: vital signs (pulse rate, blood pressure and temperature); electrocardiograms; biochemistry, hematology and urinalysis.

Assays

GH (μg/liter), PRL (μg/liter), and insulin (mU/liter; 1 mU/liter = 7.175 pmol/liter) levels were determined by use of a non-isotopic, automatic chemiluminescence immunoassay system (Immulite, DPC Inc., Los Angeles, CA). The intra- and inter-assay coefficients of variation (CV) for GH, PRL and insulin were 6.0%, 5.7%, 4.4% and 6.2%, 6.4%, 5.9%, respectively. Glucose (mmol/liter; 1 mmol/liter = 18.015 mg/dl) was measured with an automatic hexokinase method (Roche, Almere, the Netherlands). Serum IGF-I (μg/liter) was determined with a commercially available non-extraction IRMA (Diagnostic Systems Laboratories, Inc., Webster, Texas; intra- and interassay CV's, 3.9% and 4.2%, respectively).

In vitro studies

Two patients underwent transphenoidal surgery 3 months before they entered the study. Adenomatous tissue was collected during operation and subsequently, pituitary adenoma cells were isolated as described previously (17). The viability of the resulting cell suspension, as determined by trypan blue dye exclusion, was greater than 95%. The cells

were cultured at a density of 0.5-1 x 10^5 cells/dish·1 ml in multiwell plates (Corning Costar, Cambridge, MA). The culture medium was Eagle's Minimum Essential Medium with Earle's salts supplemented with a 1-fold excess of nonessential amino acids, 1mM sodium pyruvate, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml) and 10% fetal calf serum (Invitrogen, Breda, the Netherlands). Media and supplements were obtained from Gibco Bio-Cult Europe (Invitrogen, Breda, the Netherlands). The cells were allowed to attach for at least 3 days before 72-hrs incubation with SRIF (Sigma, St. Louis, MO), octreotide or SOM230 (both donated by Novartis, Basel, Switzerland) in 1 ml complete culture medium were performed with the attached cells, using four dishes for every treatment group. These pituitary cell cultures are primary cultures which were plated immediately after the isolation, and they were not passaged before the incubation studies were carried out. The results of each experiment were expressed as nanograms per dish and compared with control untreated dishes.

Quantitative RT-PCR

Quantitative RT-PCR was performed as previously described (18). Briefly, poly A^+ mRNA was isolated during Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway) from adenoma cell pellets containing 0.5-1x10^6 cells per sample. cDNA was synthesized using the poly A^+ mRNA captured on the Dynabeads Oligo $(dT)^{25}$ as a solid phase and first primer. In order to quantify sst_2 and sst_5 mRNAs a quantitive RT-PCR was performed by TaqMan® Gold nuclease assay (The Perlin-Elmer Corporation, Foster City, CA) and the ABI PRISM® 7700 Sequence Detection System (The Perkin-Elmer Corporation) for real-time amplification, according to the manufacturers instructions. The specific primer sequences (Biosource, Nivelles, Belgium) that were used include: sst_2 forward 5'-TCGGCCAAGTGGAGGAGAC-3'; sst_2 reverse 5'-AGAGACTCCCCACACAGCCA-3'; sst_5 forward 5'-CATCCTCTCCTACGCCAACAG-3'; sst_5 reverse 5'-GGAAGCTCTGGCGGAAGTT-3'; hypoxanthine-guanine phosphoribosyl transferase [(HPRT) as a control] forward 5'-

TGCTTTCCTTGGTCAGGCAGTAT-3'; HPRT reverse 5'-TCAAATCCAACAAAGTCTGGCTTATATC-3'. The probe sequences that were used included: sst_2 5'-FAM-CCGGACGGCCAAGATGATCACC-TAMRA-3'; sst_5 5'-FAM-CCCGTCCTCTACGGCTTCCTCTCTGA-TAMRA-3'; HPRT 5'-FAM-CAAGCTTGCGACCTTGACCATCTTTGGATAMRA-3'. The amount of sst_2 and sst_5 mRNA was determined by means of a standard curve generated in each experiment from known amounts of human genomic DNA. For the determination of the amount of HPRT mRNA, a standard curve was obtained by including dilutions of a pool cDNAs known to contain HPRT. The amount of sst_2 and sst_5 mRNA was calculated relative to the amount of HPRT and is given in arbitrary units.

Statistical analysis

The assumption of normality of all in vivo data was investigated by use of a Kolmogorov-Smirnov (KS) test, in which the null hypothesis that the data represented a random sample from the normal distribution was tested. When this hypothesis was not rejected, a paired Student t-test was used for assessing the statistical significance compared to the control day. The Wilcoxon's signed rank test, a non-parametric analog to the paired t-test, was used when data did not represent a random sample from normal distribution. Correlation analysis was performed by the use of Spearman's rank correlation test. In the in vitro studies, one way analysis of variance was used. When significant overall effects were obtained by this method, comparisons were made using Newman-Keuls multiple comparison test. Data are expressed as mean ± sem. A P value less than 0.05 was considered significant.

Results

Safety and tolerability

Tolerability of octreotide and SOM230 was good. Local reactions at the injection site were not observed. Side effects probably related to the study drug were reported in three different patients and were mild (one case of palpitation and sweating after SOM230 100μg, one case

of abdominal discomfort after octreotide 100μg and after SOM230 250μg). No clinically relevant changes in vital signs, routine chemistry and urinalysis were observed. Electrocardiogram analyses showed no newly occurring or worsening of known cardiac abnormalities two and 24 hrs after injection with octreotide or SOM230.

In vivo studies

Figure 1 depicts the mean circulating 24 hrs GH concentrations following a single s.c. injection of 100μg octreotide, 100μg SOM230 and 250μg SOM230, compared to control day, for all acromegalic patients investigated. Since all three treatment options appeared to induce their effect on GH secretion predominantly immediately after s.c. injection, efficacy analysis of octreotide and the two dosages of SOM230 was assessed by analysis of the mean GH suppression between 2-8 hrs after s.c. injection, compared to the same period on the control day. The mean GH levels from 2-8 hrs after SOM230 250μg, SOM230 100μg and octreotide, were suppressed by 61 ± 6.7% ($P < 0.0001$), 38 ± 7.7% ($P < 0.001$) and 59 ± 9.2% ($P < 0.0001$), respectively. Furthermore, the 250μg dosage SOM230 induced a significantly greater suppressive ef

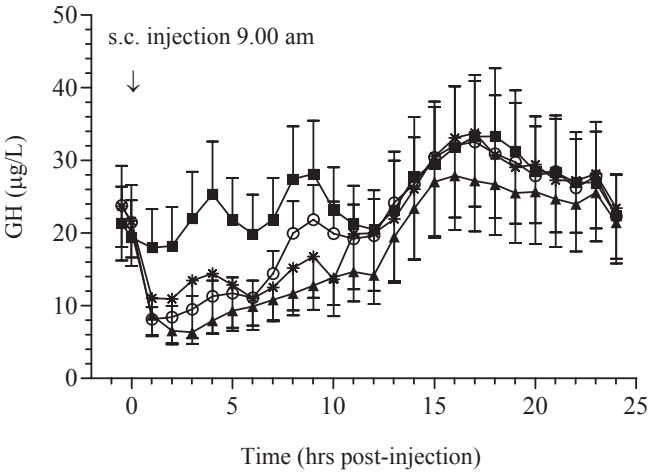

Figure 1. 24 hrs GH-concentration curves on the control day (■) and on treatment days after s.c. injection of octreotide 100μg (*), SOM230 250μg (▲) and SOM230 100μg (○). Data are expressed as mean ± sem (n=12).

fect on circulating GH concentrations than the 100µg dosage SOM230 (P < 0.01). The inhibitory effect of octreotide on GH levels did not differ from SOM230 250µg (P = ns), whereas compared to 100µg SOM230 a stronger suppression of GH concentrations by octreotide was found, although this difference failed to reach statistical significance (P = 0.13).

Analysis of the twelve individual 24 hrs GH profiles on control day and following administration of the study drugs, revealed three different patterns of response. As illustrated by the 24 hrs GH profile of patient 6 (Fig. 2A), both octreotide and SOM230 250µg induced a similar inhibitory effect on circulating GH concentrations [-63%, plasma GH levels 18.9 ± 1.1 µg/L after octreotide (*P* < 0.05) and -65%, plasma GH levels 17.7 ± 1.7 µg/L after SOM230 (*P* < 0.01), both *vs.* 50.8 ± 4.5 µg/L on control day (CD)]. A comparable suppressive effect on GH levels by octreotide

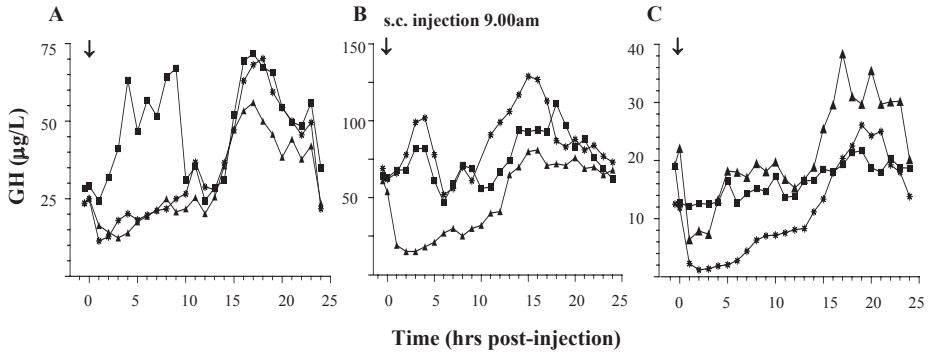

Figure 2. 24 hrs GH-concentration curves of the different response patterns after s.c. injection of the study drugs, represented by patient 6 (A), patient 12 (B) and patient 8 (C). Symbols display control day (■), octreotide 100µg (*) and SOM230 250µg (▲).

and SOM230 250µg was observed in a total of 8 patients. In this subgroup of 8 equal responders, both SOM230 and octreotide significantly suppressed GH levels by 65 ± 7% (8.0 ± 2.7 µg/L *vs.* 20.4 ± 6.5 µg/L on CD, *P* < 0.05) and 72 ± 7% (7.5 ± 3 µg/L *vs.* 20.4 ± 6.5 µg/L on CD, *P* < 0.05; octreotide *vs.* SOM230, *P* = ns), respectively (Fig. 3A). The second pattern of response to the study drugs, observed in a subgroup of three patients, is illustrated by the 24 hrs GH profile of patient 12 (Fig. 2B). In this particular patient, no decline in circulating GH

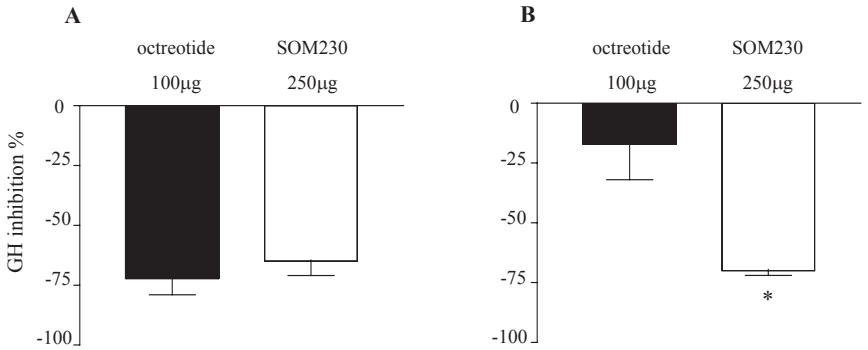

Figure 3. GH suppression 2-8 hrs after s.c. injection. The bars represent mean ± sem percentual GH suppression induced by octreotide 100µg (black bars) and SOM230 250µg (white bars) compared to the control day. A) Group showing equal response to octreotide and SOM230 (n=8). B) Group showing higher sensitivity to SOM230 (n=3; *, P < 0.05).

concentrations after octreotide administration was seen (mean plasma GH level 76.7 ± 7.3 µg/L *vs.* 67.4 ± 4.8 µg/L on CD). However, a significant suppression of serum GH levels after administration of SOM230 was found (-68%; 21.9 ± 2.2 µg/L *vs.* 67.4 ± 4.8 µg/L on CD, $P < 0.05$; SOM230 *vs.* octreotide, $P < 0.01$) and interestingly, in this particular patient the observed potent suppression by the high dose SOM230 250µg was also achieved by the low dose SOM230 100µg (-64%; 24.4 ± 3.4 µg/L). As shown in figure 3B, the mean suppression of GH levels in these three patients by SOM230 was significantly greater than the suppressive effect by octreotide [-70 ± 2%, 9.7 ± 6 µg/L and -17 ± 15%, 30.9 ± 23 µg/L, resp. *vs.* 30.5 ± 19 µg/L on control day (SOM230 *vs.* CD, $P < 0.01$; SOM230 *vs.* OCT, $P < 0.05$ and OCT *vs.* CD, P = n.s.)].

Patient 8 demonstrated a third observed response pattern (Fig. 2C), which showed a significant inhibition by octreotide (-79%, mean GH level 2.9 ± 0.7 µg/L *vs.* 13.8 ± 0.6 µg/L on CD, $P < 0.01$). SOM230 was not effective during the full 2-8 hrs post-injection time interval to elicit an inhibitory effect on circulating GH concentrations (14.5 ± 1.9 µg/L *vs.* 13.8 ± 0.6 µg/L on CD, P = n.s.). However, this patient was not insensitive to SOM230, since a short lasting suppressive effect of SOM230 was established (-40%, mean GH level 1-3 hrs after administration 7.2 ± 0.4 µg/L

vs. 12.4 ± 0.2 µg/L on CD, $P < 0.001$). Still, in this short period of time, octreotide induced a more powerful 87% suppression of GH concentrations (1.6 ± 0.3 µg/L; OCT *vs.* CD, $P < 0.001$ and OCT *vs.* SOM230, $P < 0.001$). PRL levels of all twelve patients were within the normal range [mean of 5 blood samples below 25 µg/L (men) or 44 µg/L (women); Table I]. In two patients plasma PRL levels decreased after s.c. injection of octreotide as well as with SOM230 (data not shown). Interestingly, the 24 hrs circulating plasma curves of GH and PRL levels in one of these patients was highly correlated on control day and at treatment days with octreotide and SOM230 (r_s = 0.77, 0.99 and 0.95, resp., all $P < 0.001$), which suggests a mixed GH/PRL-secreting pituitary adenoma that co-secreted both hormones from the same adenoma cell.

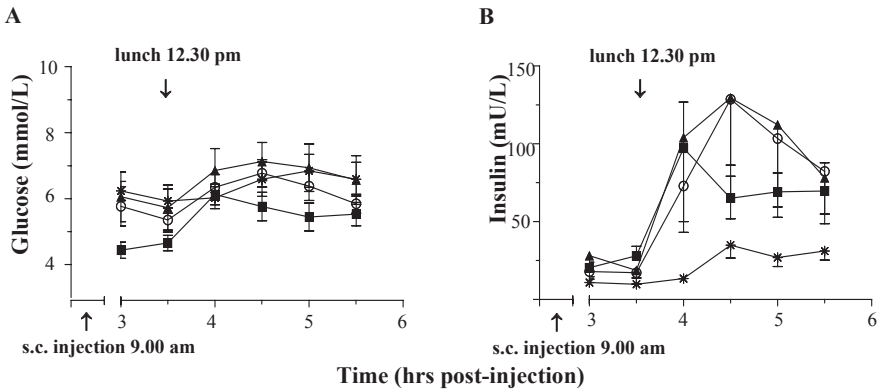

Figure 4. Mean (± sem) serum glucose (A) and insulin (B) profiles of 11 patients (one patient was excluded because of insulin-treated type II diabetes) during control day (■) and on treatment days after s.c. injection of octreotide 100µg (*), SOM230 250µg (▲) and SOM230 100µg (○).

Figure 4 shows the mean glucose and insulin concentrations of 11 patients (patient 4 was excluded since he is an insulin-treated patient with type II diabetes), starting three hrs after s.c. administration until two hrs after lunch compared to the same period of time on control day. Compared to the mean glucose level at 12:00 a.m. (3 hrs post-dose) on control day (4.4 ± 0.2 mmol/L), elevated glucose levels were observed after octreotide (6.2 ± 0.3 mmol/L, $P < 0.05$), SOM230 250µg (6.1 ± 0.8 mmol/L, $P < 0.05$) and SOM230 100µg (5.8 ± 0.6 mmol/L, $P < 0.05$) administration (Fig.

4A). When no study drug was administered, lunch induced a physiological increase in mean glucose levels to a maximum of 6.1 ± 0.3 mmol/L. Similar postprandial responses were observed on all three treatment days (octreotide, 6.8 ± 0.5 mmol/L; SOM230 250µg, 7.1 ± 0.6 mmol/L and SOM230 100 µg, 6.8 ± 0.4 mmol/L). The highest plasma glucose levels were 10.8 and 13.1 mmol/L 5 hrs after injection of octreotide and SOM230 250µg, respectively, and were both observed in patient 1, who was known to have an impaired glucose tolerance (assessed by oGTT before start of the trial). Overall, there was a trend that octreotide and both SOM230 dosages induced a comparable increase in mean glucose levels, that responded equally to a meal at 12:30 p.m. compared to control day. Octreotide induced an inhibitory effect on mean plasma insulin levels compared to control day which sustained until two hrs after lunch (1200-1430 h; $P < 0.05$). Mean insulin levels seemed not to be affected by both SOM230 dosages, since also after lunch a similar increase in insulin levels was observed as on control day (Fig. 4B). Patient 12 had severe insulin resistance. On the control day, 30 minutes after lunch was consumed, a sharp increase in plasma insulin levels to a maximum 348 mU/L was found. This was even more pronounced after SOM230 250µg was administered, when insulin levels rose up to a maximum of 903 mU/L (one hour after lunch). However, the blood glucose concentrations of this patient remained within the range of the other non-diabetic patients (Fig. 4A).

In vitro studies

Apart from the direct effects of octreotide and SOM230 on GH release by cultured pituitary tumor cells from two patients, the native peptide SRIF was also tested. GH production in the control wells from the adenoma cells of patient 6 and 12 after 72 hrs incubation amounted to 228 ± 40 ng/dish and 312 ± 18 ng/dish, respectively. In agreement with the in vivo response of patient 6 and 12 (Fig. 2A and 2B, resp.), 10 nM SOM230 lowered significantly GH secretion by -32.7 ± 6.8% and -23 ± 6.9% in the primary tumor cell cultures of patient 6 and 12, respectively ($P < 0.05$ in both instances), whereas 10 nM octreotide only inhibited

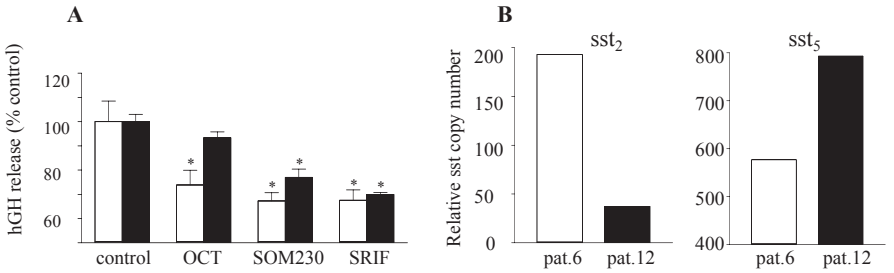

Figure 5. In vitro data of two patients. Percentual inhibition of GH secretion by 10 nM of octreotide (OCT), SOM230 and SRIF compared to control, after 72 hrs incubation in primary cultured pituitary adenoma cells from patient 6 (white bars) and 12 (black bars). Data are expressed as mean ± sem; *, $P < 0.05$ treatment vs. control (A). Quantitative analysis of RT-PCR showing the different amount of sst_2 and sst_5 mRNAs in the adenoma tissues of patient 6 and 12 (pat.6 and 12, resp.), calculated relative to the amount of HPRT and given in arbitrary units (B).

the GH secretion in the adenoma cells of patient 6 (-26.1 ± 10.5%; $P < 0.05$; Fig. 5A). SRIF lowered GH secretion in both primary cultures as well (-32.4 ± 8.5% and -30.1 ± 1.8%, resp; $P < 0.05$, Fig. 5A). Furthermore, evaluation of the relative mRNA expression levels for sst_2 and sst_5 in both cases, revealed an interesting difference. The adenoma cells from patient 6 who responded to all three compounds, had a relatively high expression of sst_2 (193 copies/HPRT) and a relative low expression of sst_5 (577 copies/HPRT). Compared with mRNA expression levels in the adenoma cells of patient 12, which were only responsive to SOM230 and SRIF treatment, an opposite mRNA expression pattern was found (Figure 5B). The pituitary adenoma of patient 12 contained relatively high mRNA expression levels for sst_5 (793 copies/HPRT) and approximately 5-fold lower sst_2 mRNA expression levels than those of patient 6 (37 copies/HPRT). The adenoma was in vivo and in vitro not responsive to octreotide, but demonstrated significant sensitivity to both dosages SOM230 in vivo and to SOM230 in vitro. This suggests the involvement of sst_5 subtype in the GH-release inhibitory effect in this particular case.

Discussion

In the present study, the recently developed SRIF-analog SOM230, exhibiting an universal binding profile which was demonstrated to effec-

tively suppress GH levels in normal monkeys and rodents (15), was administered for the first time in acromegalic patients in order to assess its efficacy in comparison to octreotide. The acute effects of a single dose of SOM230 250µg and octreotide 100µg on circulating GH concentrations demonstrated three patterns of response in the 12 patients investigated. In 8 patients both SRIF-analogs were equally effective in lowering GH levels. This suggests that in these patients the sst_2 subtype is the major receptor on the pituitary adenoma which is responsible for mediating these inhibitory effects. It is well known that sst_2 especially is involved in the inhibitory actions of SRIF and SRIF-analogs on hormone secretion, both in primary cultured human fetal pituitary cells and GH-secreting pituitary adenoma cells as well (11, 19). The relative amount of mRNA expression levels of this receptor subtype was positively correlated with the sensitivity to octreotide treatment *in vitro* (20). The *in vitro* data of patient 6 illustrate in this group of equal responders, that the relatively high mRNA level for sst_2 combined with the good affinity of both SOM230 and octreotide account for the suppressive effects of both drugs on GH secretion in this group of patients. SOM230 has a 2.5 times lower affinity to sst_2 than octreotide, which explains the similar effect of 250µg SOM230 and octreotide 100µg in this category of acromegalic patients.

The second pattern of response, illustrated by patient 12 in which SOM230 is far more efficacious compared to octreotide in suppressing GH levels, was observed in three patients. A pivotal role for sst_5 in mediating suppression of GH release is probable. The *in vitro* data of patient 12 show relatively low mRNA expression levels for sst_2 and higher expression levels for sst_5. The median inhibitory concentration (IC_{50}) for sst_5 of SOM230 is 0.16 nmol/L and that of octreotide is 40-times higher (6.3 nmol/L), pointing to the higher affinity of SOM230 for the sst_5 subtype. These observations, together with the the *in vitro* significant inhibition by the native SRIF on the primary culture of adenoma cells, suggest that both SRIF and SOM230 exert their potent effects in this particular tumor via sst_5 subtype. So far, the role of sst_5 in mediating GH release was only investigated in studies with primary cultures of pituitary adenoma cells

obtained from acromegalic patients (19, 20). Saveanu and his group found a 30-fold higher expression of sst_5 compared to sst_2 mRNA in four adenomas poorly responsive to octreotide. The addition of the sst_5 specific analog BIM23268 to the medium achieved a maximal GH suppression. This suggests a "rescue" through sst_5 when tumors are only partial sensitive to octreotide (14). SOM230 induced a 3-fold stronger inhibition than octreotide on GH release by cultured rat pituitary cells and a pronounced inhibition of plasma IGF-I levels in rodents after 18 weeks of treatment, which again is suggestive for sst_5 involvement (15). We present the first clinical evidence that the sst_5 subtype may indeed play an essential role in mediating the *in vivo* suppressive actions by SOM230 on GH concentrations in three acromegalic patients, which were (partially) unresponsive to octreotide. Since SOM230 is able to lower GH levels in both subgroups of patients, coupled to sst_2 and sst_5 subtype physiology, respectively, this novel SRIF-analog has a clear advantage over octreotide and might increase the number of patients which can be biochemically controlled during long-term medical treatment. Furthermore, in patient 12, 100µg and 250µg of SOM230 suppressed GH levels equally. In this particular case, increasing the SOM230 dosage by a factor 2.5 did not result in a further increase in GH inhibition. This phenomenon is already known for patients who are sensitive to octreotide treatment: a similar GH suppression is found upon s.c. injections with octreotide dosages in the range of 100-1500 µg/day (21, 22). This could indicate that the density of the predominantly expressed sst determines the response to a SRIF-analog: in GH-secreting adenomas expressing sst_2 in high density, octreotide is able to suppress GH levels significantly. However, if sst_2 is almost not expressed on the pituitary adenoma, sst_5 mediates the GH-suppressive effects of SRIF and SRIF-analogs. The dose-response curves of octreotide and SOM230 seem to reach the plateau at low levels when high densities of sst_2 and sst_5, respectively, are expressed. *In vivo* and *in vitro* data from this trial emphasize that the inhibitory effects on GH release by SRIF and its analogs are primarily mediated via sst_2, as seen in the group of 8 equal responders to octreotide and SOM230. However, when sst_2 over sst_5 mRNA levels are being expressed below a certain threshold as in patient number 12, a

suppressive action upon GH concentrations via sst_5 receptors becomes visible. In addition, heterodimeric effects of different sst subtypes are suggested to play a role in receptor physiology (23, 24) and as discussed before, the BIM23244 bispecific sst_{2+5} analog has already shown to be more active than the combination of a sst_2 specific analog combined with a sst_5 specific analog on GH release (14, 25), indicating that a heterodimeric effect by SOM230 on sst_2 and sst_5 subtypes cannot be ruled out.

The third response was observed in one patient, who only transiently responded to SOM230, whereas octreotide was far more efficacious in lowering GH levels. The most likely explanation is the presence of a relatively high sst_2 and a low sst_5 mRNA expression level, resulting in a high sensitivity for octreotide. Whether higher dosages of SOM230 would indeed induce similar lowering actions on GH concentrations as seen by octreotide, remains uncertain. Since SRIF and its analogs inhibit the secretion of insulin, impaired postprandial glucose tolerance was observed after the acute administration of octreotide (26). Similar elevations of glucose concentrations were observed after SOM230. However, the elevated glucose levels seem not be caused by an inhibitory action on insulin release, since after SOM230 administration at 09:00 a.m. an almost identical insulin response was observed after lunch as on the control day. At present, the mechanism of this transient increase in glucose levels remains uncertain. Several studies support a role for sst_5 to control insulin secretion in rats, mice and humans, whereas sst_2 mediates glucagon secretion from the pancreatic α-cells (27-30). On the basis of the SOM230 and octreotide affinity profiles for sst_2 and sst_5, it seems unlikely that octreotide, binding 40-fold less to sst_5 compared to SOM230, would exert such a strong and long lasting insulin inhibition via sst_5 subtype whereas SOM230 treatment resulted in barely any inhibition. Therefore, these opposed effects of octreotide and SOM230 on insulin levels, suggest a pivotal role for sst_2 subtype in regulating human insulin secretion. In cynomolgus monkeys, insulin, glucagon and glucose levels remained unchanged during seven days of high-dose infusion with SOM230. Furthermore, during

an 18-week treatment with pharmacological doses of SOM230 plasma glucose levels were not changed, indicating that SOM230 is well tolerated in rats and monkeys with regard to glucose homeostasis (15, 31). The promising pharmokinetic properties of SOM230 found *in vivo* in rats, accounting for a terminal elimination half-life of 27 hrs compared to 2 hrs for octreotide (15), did not result in a longer duration of action of SOM230 than that of octreotide. Probably, serum SOM230 concentrations drop sooner below a certain therapeutical level, leading to a duration of action on GH levels comparable to that of octreotide treatment. In conclusion, our data suggest that SOM230 has the potency to increase the number of acromegalic patients which can be biochemically controlled during long-term medical treatment, since it's additional suppressive effects on GH secretion via sst_5. However, the subtype sst_2 seems to be the dominant receptor in controlling hypersecretion in acromegaly. No serious side effects occurred during SOM230 treatment. The subtle increase in glucose levels after SOM230 injection needs further attention and can not be explained by sst_5 or sst_2 mediated action on insulin secretion. Future studies will also address the question whether SOM230 can control pituitary adenoma size in acromegaly better than octreotide (8). Besides sst_2 and sst_5, sst_1 and sst_3 also seem to be involved in cell proliferation and in the induction of apoptosis (32-35). This suggests that the universal SRIF-analog SOM230, with good affinity for both sst_1 and sst_3, might have possible antiproliferative and tumor size reducing effects as well.

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Chapter III-3

The somatostatin analog SOM230, compared with octreotide, induces differential effects in several metabolic pathways in acromegalic patients

Clin Endocrinol. 2005, 63 (2): 176-84

Abstract

OBJECTIVE Recently, our first clinical study with the novel multi-ligand somatostatin (SRIF) analog SOM230 in acromegalic patients showed that SOM230, due to its beneficial inhibitory effects on GH levels compared with octreotide (OCT), might increase the number of patients that can be biochemically controlled. Since SRIF-analogs are also known to interact with other metabolic pathways, assessment of IGF-I, IGFBP-1, glucose and insulin concentrations on the control day (CD) and on treatments days following a single s.c. injection SOM230 100 and 250µg, compared with OCT 100µg, was performed.

DESIGN AND PATIENTS Randomized, cross-over, double-blinded proof-of-concept trial in 12 patients with active acromegaly.

RESULTS Total IGF-I levels, compared with predose, were not affected. Free IGF-I levels were suppressed after 24h by OCT, SOM230 250 and 100µg, whereas at 48h only both SOM230 dosages still induced these inhibitory effects. Circulating IGFBP-1 levels (AUC; 08.30-14.30h) compared with CD, increased sharply after OCT (from 48 to 237 µg/Lh; $p<0.001$ vs CD), while SOM230 250 and 100µg elicited a lower and dose-dependent effect (163 and 90 µg/Lh, resp; $p<0.05$ vs CD and OCT). Neither insulin nor GH levels showed statistically significant correlation with IGFBP-1 levels either after SOM230 or OCT. An early rise in glucose levels 1h post-injection with SOM230 250µg compared with OCT and CD was observed 8.3±0.8, 4.4±0.5 and 4.9±0.4 mmol/L, resp: $p<0.05$). SOM230 250µg (19±4 vs 46±3 mU/L on CD: $p<0.05$), although clearly less potent than OCT (5.4±0.4 mU/L: $p<0.01$ vs CD), inhibited insulin release. Since these corresponding absolute insulin levels cannot entirely explain this hyperglycaemic effect of SOM230, other mechanisms seem involved in this glucose rise. If SOM230 would influence glucose homeostasis in peripheral target tissues of insulin action, expression of SRIF-receptors (sst) seems a logical necessity. In normal human liver tissues, analyzed by quantitative PCR, the average sst_1 mRNA expression level appeared significantly higher compared with sst_2 (n=6, relative copy number 161±46 vs 57±6; $p<0.05$). Fat tissue expressed both sst_1 and sst_2 mRNA, whereas in muscle only sst_2 mRNA was found.

CONCLUSION Both dosages SOM230 inhibit free IGF-I more sustained

as compared with OCT, implying longer duration of action. The superior action of OCT compared with SOM230 in stimulating IGFBP-1 levels, suggests direct regulation of IGFBP-1 by SRIF-analogs via sst_2. Finally, expression of only sst_1 and sst_2 in target tissues of insulin action, might point towards additional modulatory effects by SOM230 on glucose homeostasis.

Introduction

Acromegaly is a rare disease characterized by chronic hypersecretion of growth hormone (GH), which is almost exclusively caused by a GH-secreting pituitary adenoma. The clinical manifestations of acromegaly are due to the peripheral actions of excess GH and insulin-like growth factor-I (IGF-I). Successful medical treatment of acromegaly with the current clinically available somatostatin (SRIF) analogs octreotide (OCT) and lanreotide is due to the expression of high-affinity (density) SRIF receptors on the adenoma cells, mainly SRIF receptor subtype (sst) 2, which is one of the five known SRIF receptor subtypes (1-4), sst_1-sst_5, and the selective high-affinity binding of these analogs to sst_2 (Table I). Several

Table I. Binding affinity of OCT and SOM230 for the five human sst, $sst_{1-5.}$

compound	sst_1	sst_2	sst_3	sst_4	sst_5
OCT	280 ± 80	0.38 ± 0.08	7.1 ± 1.4	> 1000	6.3 ± 1.0
SOM230	9.3 ± 0.1	1.0 ± 0.1	1.5 ± 0.3	> 100	0.16 ± 0.01

Data are reproduced with permission (11). Results are the mean ± sem; IC50 are expressed in nanomolar concentration.

studies with this generation of octapeptide SRIF analogs have demonstrated that long-term therapy, administered subcutaneously (s.c.) or as a long-acting depot preparation, induces clinical and biochemical "cure" in up to 65% of patients (5-8). Because approximately one third of patients with acromegaly seem (partially) resistant to OCT and/or lanreotide administration, improved compounds to treat acromegaly are required. The recently identified novel synthetic SRIF-peptidomimetic SOM230 forms

a serious candidate to obtain such therapeutic significance in acromegaly (9). Compared with OCT, SOM230 has a 30, 5 and 40 times higher binding affinity to sst_1, sst_3 and sst_5, respectively, and 2.5 times lower affinity to sst_2 (Table I). Preclinical studies showed that SOM230 potently and dose-dependently suppressed GH and IGF-I levels for prolonged periods of time up to 120 days in rats, dogs and rhesus monkeys (10, 11). Additional characteristics of SOM230 include a favourable terminal elimination half-life of 24 h in humans as well as the preliminary evidence that glucose levels in rats and dogs remain normal during long-term administration of the compound (10). Clinical evaluation of SOM230, by assessing its efficacy compared with OCT, was recently performed in a single dose proof-of-concept trial in acromegaly (12). It was concluded that SOM230, due to its beneficial effects on circulating GH levels compared with OCT, might increase the number of patients that can be biochemically controlled during long-term medical treatment. The advantage of SOM230 compared with the current clinically available sst_2-preferring analogs, likely resulted from its sst_5-mediated suppressive effects on GH levels in a subgroup of patients that were (partially) unresponsive to OCT administration.

However, since SRIF and its analogs inhibit the secretion of insulin (13), impaired postprandial glucose tolerance was observed after the acute administration of OCT (12). Comparable elevations of glucose concentrations were observed after SOM230, whereas SOM230 did not alter the meal-induced insulin response (12). Therefore, the elevated glucose levels seem not to be caused by an inhibitory action on insulin release. Still, the mechanism of this early transient increase in glucose levels remains uncertain. In order to obtain more insight with respect to the acute effects of SOM230 on carbohydrate metabolism in active acromegaly, additional analysis of insulin, glucose and insulin-like growth factor binding protein (IGFBP) 1 levels of the 12 acromegalic patients that participated in the proof-of-concept trial, was conducted. Furthermore, we present the first clinical data regarding the effects of a single dose SOM230 on total and free IGF-I levels in acromegaly.

Methods

Patients

Twelve patients with active acromegaly were recruited at the Erasmus MC Rotterdam, The Netherlands. Patient characteristics have been described in detail previously (12). All subjects had biochemically active disease, with a mean serum GH concentration greater than 5 μg/L during a 5-hour profile and elevated circulating IGF-I levels (age- and sex-adjusted). GH concentration failed to suppress below 1 μg/L after a 2-hour 75-g oral glucose tolerance test (oGTT). Seven patients had been treated before (see below). In those patients who have been medically treated previously, a wash-out period after the last dose of medication had to be at least 1 month, 1 week, 4 months and 1 month for dopamine agonists, subcutaneous formulations of octreotide, depot formulations of long-acting somatostatin analogs and growth hormone receptor antagonists, respectively. One patient had been previously treated by surgery, medical treatment and irradiation. One patient was treated only with surgery. Two patients had only been medically treated and three patients were treated with surgery and medical treatment. Five patients were newly diagnosed. Pituitary function was preserved in 8 patients. Gonadal steroid-, glucocorticoid- as well as thyroxin-replacement therapy was initiated in 3 patients before start of the study, whereas 1 patient was treated with only gonadal steroids, Patients with compression of the optic chiasm causing any visual field defect or those requiring surgical intervention for relief of any sign or symptom possibly associated with tumor compression, were excluded. The local ethical committee of the Erasmus MC approved the study and all patients gave written informed consent.

Treatment protocol

Patients were hospitalized on the control day for 24 h for the assessment of baseline efficacy parameters. On study day 1, 8 and 15, each patient received at 09:00 a.m. a single s.c. injection of OCT 100μg, SOM230 100μg or SOM230 250μg in a randomized, double-blinded, crossover design with a minimum 6 days of washout between drug treatments. All pa-

tients received standardized meals, served at 08:30 a.m., 12:30 p.m. and 17:30 p.m. In addition to the recently published data (12), blood samples, initially centrifuged and immediately frozen at -20°C, for the assessment of insulin, glucose and IGFBP-1 concentrations were collected at the following time points: 30 minutes and one minute before, and every hour for 2 h after drug administration. Furthermore, blood samples for IGFBP-1 assessment were collected 30 minutes and one minute before, and every half hour for 2 h after lunch. Finally, for the assessment of total and free IGF-I levels, blood samples were collected 30 minutes before, 24 and 48 h after s.c. injection. These procedures were repeated on all study days.

Assays

Insulin (mU/L; 1 mU/L = 7.175 pmol/L) levels were determined by use of a non-isotopic, automatic chemiluminescence immunoassay system (Immulite, DPC Inc., Los Angeles, CA). The intra- and inter-assay coefficients of variation (CV) for insulin were 4.4% and 5.9%, respectively. Glucose (mmol/L; 1 mmol/L = 18.015 mg/dl) was measured with an automatic hexokinase method (Roche, Almere, the Netherlands). Serum total IGF-I (nmol/L), free IGF-I (pmol/L) and IGFBP-1 (μg/L) were determined with a commercially available non-extraction IRMA (Diagnostic Systems Laboratories, Inc., Webster, Texas). All assays were carried out in a blinded manner, and quality-control samples were included within assay runs. Interassay coefficients of variation for total IGF-I, free IGF-I, and IGFBP-1 were 4.2%, 5.1 and 6.0%, respectively. Intraassay coefficients of variation for total IGF-I, free IGF-I and IGFBP-1 were 3.9, 5.1 and 4.6%, respectively.

RNA isolation

Sst mRNA expression in peripheral insulin-targeted tissues was analyzed. Per-operatively obtained human liver biopsies from 6 patients diagnosed with hepatocellular carcinoma (n=3), colorectal carcinoma (n=2) or hemangioma (n=1), were collected. Collected liver tissues were directly frozen and stored at –80 °C until pathological examination confirmed that the biopsies showed normal liver tissue histology. Visceral adipose

tissue was obtained from two organ-transplant donors, directly frozen and stored at –80 °C, who were initially admitted at Leiden University Medical Center, The Netherlands. The protocols were in accordance with the Helsinki Doctrine on Human Experimentation. Informed consent was obtained from the patient or from the closest family member. Collected tissues were grinded to powder on dry ice using a mortar and total RNA was isolated using either a High Pure RNA Tissue Kit for tissue samples (Roche Diagnostics GmbH, Mannheim, Germany) or a RNeasy® Lipid Tissue Mini Kit for adipose tissue samples (QIAGEN, Westburg B.V., Leusden, The Netherlands) according to manufacturers protocol. cDNA from two skeletal muscle (rectus abdominis) biopsies was kindly provided by Dr. van den Berghe (Department of Intesive Care Medicine, Catholic University of Leuven, Belgium) from two critically ill patients that participated in a large randomized, controlled study on intensive insulin treatment in Intensive Care Unit patients, of which the major clinical outcome have been published in detail elsewhere (14, 15).

Quantitative RT-PCR

Quantitative RT-PCR was performed as previously described (16). Briefly, poly A^+ mRNA was isolated during Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway). cDNA was synthesized using the poly A^+ mRNA captured on the Dynabeads Oligo $(dT)_{25}$ as a solid phase and first primer. One-twentieth of the cDNA library was used for quantification of sst subtype mRNA levels. A quantitative PCR was performed by TaqMan® Gold nuclease assay (The Perkin-Elmer Corporation, Foster City, CA) and the ABI PRISM® 7700 Sequence Detection System (The Perkin-Elmer Corporation) for real-time amplification, according to the manufacturer's instructions. The assay was performed using 15µl TaqMan® Universal PCR Master Mix (Applied Biosystems, The Netherlands), 500 nM forward primer, 500 nM reverse primer, 100 nM probe and 10µl cDNA template, in a total reaction volume of 25µl. After an initial heating at 95° C for 8 minutes, samples were subjected to 40 cycles of denaturation at 95° C for 15 seconds and annealing for 1 minute at 60° C.

The primer and probe sequences that were used for the detection of sst_1, sst_2, sst_3, sst_5 and hypoxanthine phosphoribosyl transferase (HPRT) mRNA's have been described previously (16). In addition, we also evaluated sst_4 mRNA expression in the present study using the following primers and probe: **sst_4** forward 5'-CTGCGCCAACCCTATTCTCT-3'; **sst_4** reverse 5'-ACCCGCTGGAAGGATCG-3'; **sst_4** probe 5'-FAM-TGGCTTCCTCTCCGACAACTTCCG-TAMRA-3'. Primers and probes were purchased from Biosource (Nivelles, Belgium). The relative amount of sst subtype mRNA was determined using a standard curve generated from known amounts of human genomic DNA. For the determination of the amount of HPRT mRNA, a standard curve was obtained by including dilutions of a pool of cDNAs from a human cell line known to express HPRT. The relative amount of sst subtype mRNA was calculated relative to the amount of HPRT mRNA and is given in arbitrary units. Each sample was assayed in duplicate.

Statistical analysis

The assumption of normality was investigated by use of a Kolmogorov-Smirnov test. When this hypothesis was not rejected, a paired Student t-test was used for assessing the statistical significance compared with the control day. The Wilcoxon's signed rank test, a non-parametric analog to the paired t-test, was used when data did not represent a random sample from normal distribution. Correlation analysis was performed by the use of Spearman's rank correlation test. IGFBP-1 levels were also analyzed as area under the curve (AUC) values. Quantitative RT-PCR data were analyzed by ANOVA to determine overall differences between sst subtype mRNA expression. When significant differences were found by ANOVA, a multiple comparison sst subtypes was made using the Newman-Keuls test. Data are expressed as mean ± sem. A *P v*alue less than 0.05 was considered significant.

Results

IGF-I concentrations

Total IGF-I levels, compared with predose, were not affected 24 and 48 h after OCT and SOM230 treatments (Fig. 1 upper panel). However free IGF-I levels were suppressed after 24 h by OCT 100µg, SOM230 100µg and 250µg (-30 ± 5%, -23 ± 5% and -30 ± 7%, respectively: $p<0.01$ to predose), whereby at 48 h only after both SOM230 dosages these inhibitory effects persisted ($p<0.01$ to predose; Fig. 1 lower panel).

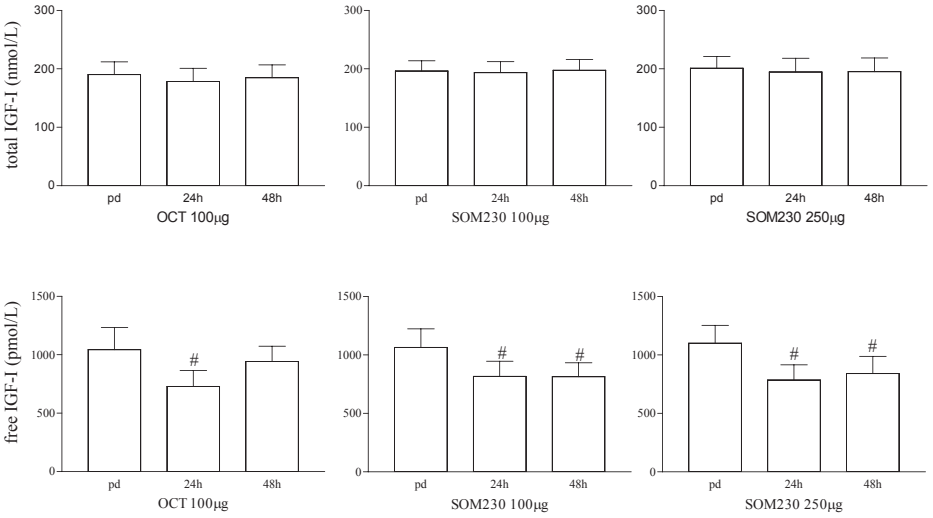

Figure 1. Total and free IGF-I levels (upper and lower panel, respectively) on treatment days after s.c injection with the study drugs. Data are expressed as mean ± sem (n=12). # $P < 0.05$ vs predose (pd).

IGFBP-1 concentrations

As shown in figure 2, OCT induced a higher response with respect to circulating IGFBP-1 levels. IGFBP-1 concentrations (AUC; 08.30-14.30 h) compared with CD, increased sharply after OCT (from 48 to 237 µg/Lh; $p<0.001$ vs CD), while SOM230 250 and 100µg elicited a lower dose-dependent effect as well (to 163 and 90 µg/Lh, respectively; $p<0.05$ vs CD: OCT vs SOM230 250 and 100µg, $p<0.05$). Neither insulin nor GH levels showed statistically significant correlation with serum IGFBP-1 concentrations either after both dosages SOM230 or OCT (Table II).

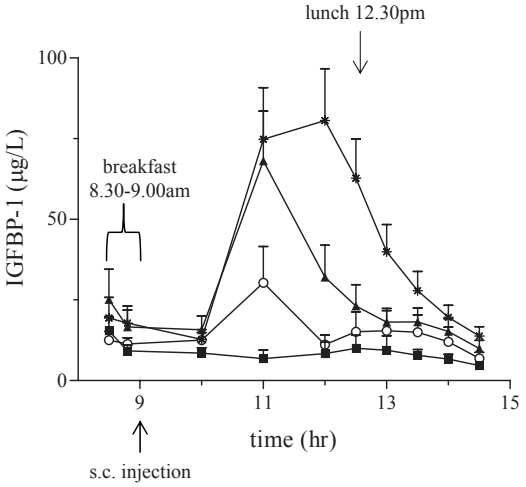

Figure 2. Serum Insulin-like Growth Factor Binding Protein 1 (IGFBP-1) concentrations following a s.c. injection with the study drugs in comparison with a control day. Values represent mean ± sem [n=11, one patient was excluded because of insulin-treated type II diabetes, (12)]. Symbols display control day (■), OCT 100µg (*), SOM230 250µg (▲) and SOM230 100µg (○).

Table II. Spearman rank correlation coefficients (rs) of IGFBP-1 with GH and insulin levels in 11 patients with active acromegaly.

Treatment	Metabolic parameter			
	GH		Insulin	
	r_s	p-value	r_s	p-value
SOM230 100µg	-0.18	0.71	-0.31	0.39
SOM230 250µg	-0.5	0.27	-0.36	0.31
OCT 100µg	-0.04	0.96	-0.32	0.37

Carbohydrate metabolism

The effects of SOM230 and OCT on carbohydrate metabolism during lunch have been described already extensively (12). Briefly, glucose levels were slightly elevated after all drug treatments compared with control day. Lunch was accompanied by a physiological increase in glucose levels on the control day and all treatment days. OCT inhibited insulin levels

until 2 h after lunch, whereas both dosages SOM230 did not statistically significantly affect insulin levels. An early raise in glucose levels 1 h post-injection with SOM230 250µg compared with SOM230 100µg, OCT and CD (8.3 ± 0.8, 6.4 ± 0.7, 4.4 ± 0.5 and 4.9 ± 0.4 mmol/L, resp: $p<0.05$) was seen (Fig. 3A). Two hours after s.c. injection glucose levels were raised during OCT treatment as well (6.3 ± 0.4 vs 4.4 ± 0.3 mmol/L on CD: $p<0.05$). At both time points OCT already suppressed insulin levels, whereas only at 1 h post-injection SOM230 100 and 250µg (18 ± 4 and 19 ± 4 vs. 46 ± 3 mU/L on CD: both $p<0.05$), although clearly less potent than OCT (5.4 ± 0.4 mU/L: $p<0.01$ vs CD), inhibited insulin release (Fig. 3B).

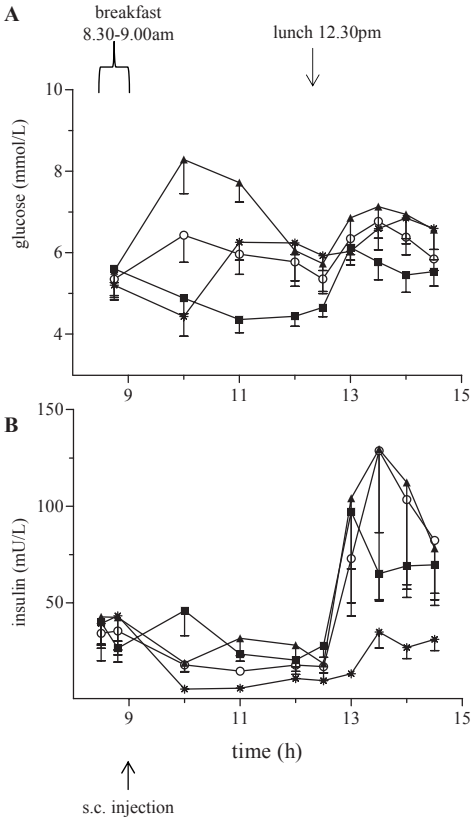

Figure 3. Mean (± sem) serum glucose (A) and insulin (B) profiles of 11 patients [one patient was excluded because of insulin-treated type II diabetes, (12)] during control day (■) and on treatment days after s.c. injection of OCT 100µg (*), SOM230 250µg (▲) and SOM230 100µg (○).Assessment of metabolic parameters in the subgroup of 8 patients, responding equally effective to 100µg OCT and 250µg SOM230 with respect to circulating GH concentrations

In order to homogenise the metabolic responses for the effects of both SS-analogs on circulating GH concentrations, we have also analysed all metabolic parameters in the subgroup of 8 acromegalic patients that responded to a similar extent to 100μg OCT and 250μg SOM230 with respect to circulating GH levels (12). Total IGF-I concentrations, compared with predose, were slightly attenuated only after 24h by 100μg OCT and 250μg SOM230 (-11 ± 2% and -9 ± 2%, resp: $p<0.05$) but not by 100μg SOM230 (-4 ± 3%). The lowering of circulating free IGF-I concentrations by OCT and both dosages SOM230 in the subgroup of 8 patients, appeared similar as compared with the total group of 12 patients, *i.e.* 100μg OCT, 100μg and 250μg SOM230 suppressed free IGF-I levels 24h after s.c. injection (-30 ± 6%, -26 ± 7% and -35 ± 6%, resp: $p<0.05$) whereby at 48 h only after 100μg and 250μg SOM230 these inhibitory effects persisted (-28 ± 8% and -26 ± 6%, resp: $p<0.05$). The differential effects of OCT and both dosages SOM230 on circulating IGFBP-1 and glucose homeostasis in the total group of 12 patients, as described above, were also observed when IGFBP-1, glucose and insulin concentrations were analyzed in the subgroup of 8 patients that responded in a similar extent to OCT and SOM230 with respect to circulating GH concentrations.

Sst mRNA expression in human liver, muscle and visceral adipose tissue

As described above, the observed raise in glucose levels after s.c administration of 250 μg SOM230 (and to a lesser extent by 100μg SOM230) was not accompanied by a profound inhibitory action of the compound on insulin secretion by pancreatic β-cells. Therefore, other (extra-pancreatic) mechanisms could be responsible for the detoriated carbohydrate metabolism after SOM230 administration. It is generally well known that liver, muscle and fat are peripheral tissues predominantly involved in the regulatory actions of insulin to control plasma glucose levels. If SOM230 influences glucose homeostasis in these target tissues directly, expression of SRIF receptors seems a logical necessity. Therefore, we evaluated by quantitative PCR, for the first time the presence of sst_1

and sst_2 mRNA in human liver, muscle and fat tissues (Figure 4). The other sst subtypes, sst_{3-5}, were not expressed. In liver, the average sst_1 mRNA expression level was significantly higher compared with sst_2 (n=6, relative copy number 161 ± 46 vs 57 ± 6; $p < 0.05$). Visceral fat tissue expressed both sst_1 (n=2, relative copy number 705 and 182) and sst_2 (n=2, relative copy number 403 and 263) mRNA, whereas in muscle only sst_2 mRNA (n=2, relative copy number 106 and 64) was found.

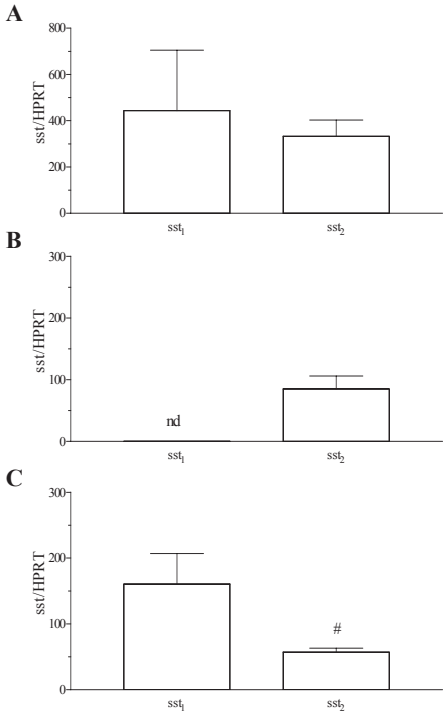

Figure 4. Quantitative analysis of RT-PCR showing the relative amounts of two SRIF receptor subtypes, sst_{1-2}, mRNA in three peripheral target tissues of insulin, calculated relative to the amount of HPRT and given in arbitrary units. A) Visceral adipose fat, n=2: B) Skeletal muscle, n=2 and C) Liver, n=6; # $p < 0.05$. nd; not detectable.

Discussion

This report is unique due to the fact that SOM230 is the first multiligand SRIF-analog administered *in vivo* to acromegalic patients. Up till now, only sst_2 preferring analogs, *i.e.* OCT and Lanreotide have been used clinically. Our study is in support of the clinical poten-

tial of novel multiligand SRIF-analogs in acromegaly, but tantalising novel insights in sst subtype physiology are demonstrated as well. No effects on total IGF-I levels after single doses of SOM230 and OCT were observed in our series of 12 acromegalic patients, which might be related to its long half-life. However, in the subgroup of 8 acromegalic patients that responded equally to OCT and 250μg SOM230 with respect to circulating GH concentrations (12), total IGF-I concentrations were lowered to a small extent 24h after s.c. injection with 100μg OCT and 250μg SOM230. The latter observation could indicate the GH-dependent inhibition of total IGF-I. However, the temporal, short-term feedback between GH secretion and the circulating IGF-I system in normal subjects, is recently suggested to be regulated more importantly due to changes in free IGF-I levels instead of total IGF-I levels (17). Interestingly, both in the total group of 12 patients as well as in the GH-equal responder group of 8 patients, single dose administrations of 100 and 250μg SOM230 persistently inhibited free IGF-I levels significantly after 48 h, whereas OCT was only effective for 24 h. This could be explained by intracellular dynamics of sst_2 and sst_5 at the central level of the pituitary (18), combined with the 40-fold higher sst_5-binding affinity and the longer half life of SOM230 compared with OCT. Stroh and coworkers demonstrated that sst_2 seems to be rapidly internalized after ligand binding, but sst_5 showed early recycling after internalization, with massive re-recruitment from intracellular stores (back) to the membrane (19). On the other hand, two other reports recently reported that sst_2 can be rapidly recycled to the membranes as well (20, 21), SOM230 might induce this sustained inhibition of free IGF-I levels via sst_2 as well because of its long half life as compared with OCT. In addition, there is compelling evidence that SRIF analogs act to suppress IGF-I by inhibiting pituitary GH release (22, 23) but whether SRIF analogs act on peripheral target tissues of GH to reduce GH-induced IGF-I production as well remains to be further clarified. Melmed's group recently demonstrated in rat hepatocytes and perfused rat livers, expressing sst_2 and sst_3, that SRIF and OCT dose-dependently inhibited GH-induced IGF-I production at the level of the hepatocyte (24). In respect to the sustained free IGF-I suppression by

SOM230 in our acromegalic patients, the question regarding the sst subtype responsible for these peripheral inhibitory effects is of importance. Based on sst binding affinity profiles of SOM230 and OCT (Table I), combined with our present data showing only sst_1 and sst_2 mRNA expression in human liver tissues, both sst subtypes might mediate peripheral IGF-I production; 1) sst_1 may be involved because SOM230, compared with OCT, has a 30-fold higher sst_1-binding affinity and 2) SOM230's favorable half-life of nearly 24 h induces prolonged activation of sst_2.

SRIF has been implicated in the regulation of IGFBP-1 (25), that blocks availability of IGF-I (26). OCT induced a potent increase in serum IGFBP-1 levels in the acromegalic patients, within 2-3 h following injection, a time course in agreement with earlier reports (27-29). Even though 100 and 250μg SOM230 administration significantly and dose-dependently increased IGFBP-1 levels, OCT treatment remained more potent with respect to IGFBP-1 release. Furthermore, we could not demonstrate a significant relation between the course of either circulating insulin or GH levels and IGFBP-1 concentrations, which has been reported previously, arguing against a direct regulatory effect of GH on IGFBP-1 production in acromegaly (27, 30, 31). Several studies have shown a statistical significant inverse correlation between serum insulin and IGFBP-1 concentrations in acromegaly and patients with insulin dependent diabetes mellitus, suggesting a regulatory effect of insulin on circulating IGFBP-1 levels (31-34), whereas others did not (27, 30). The absence of any statistically significant correlation between insulin and IGFBP-1 levels after both OCT and SOM230 administration might support a direct role for SRIF-analog induced release of IGFBP-1 in acromegaly. Evidence for a direct induction of IGFBP-1 mRNA by OCT has been observed in human hepatoma cells, but this effect was noted only after 12 h of OCT-incubation (35). Considering the superior action of OCT compared with SOM230 in stimulating IGFBP-1 levels in acromegaly, combined with the higher sst_2-binding affinity by OCT (Table I), these data point towards a modulatory role of sst_2 in the direct regulation of IGFBP-1 levels by SRIF-analogs in acromegaly.

In general, the effects of SRIF-analogs on glucose homeostasis appeared to be minor and a mild deterioration occurred only in those without impaired glucose tolerance (36). Indeed, compared with OCT, comparable elevations of glucose concentrations during lunch were observed after SOM230 administration early in the morning in acromegalic patients (12). In addition, 250μg SOM230 elicited an acute raise of glucose levels 1 h post-injection (figure 3). SOM230-mediated inhibitory effects on insulin secretion cannot explain these elevated glucose levels. Several studies support a role for sst_5 to control insulin secretion in rats, mice and humans, (37-40). On the basis of SOM230 and OCT binding affinity profiles for sst_5 (Table I), it seems unlikely that OCT, binding 40-fold less to sst_5 compared with SOM230, would exert such a strong and long lasting insulin inhibition via sst_5 subtype whereas SOM230 treatment resulted in barely any inhibition. Therefore, these seemingly discrepant effects of OCT and SOM230 on insulin levels, which represent *in vivo* novel insights in the role of sst subtypes in human pancreatic insulin secretion, clearly suggest a dominant role for sst_2 in regulating human insulin secretion. Support for this hypothesis comes from recent experiments performed with isolated perifused human pancreas tissues, which showed inhibitory effects of OCT or a specific sst_2 agonist in low near "physiological" concentrations on insulin secretion, while an sst_5 agonist inhibited insulin secretion only in "pharmacological" doses (41). As glucagon release appeared far less sensitive for SOM230 as compared with octreotide (44-fold) in several in vivo animal models and peripheral glucagon measurement does not represent pancreatic glucagon (gastro-intestinal glucagon is measured as well) concentrations, the effects of SOM230 and OCT on glucagon levels have not been analyzed. Recent immunohistochemical observations in human pancreatic tissue have shown a wide occurrence of both sst_2 and sst_5 in α-cells (42, 43), indicating that it is not clear yet whether SOM230 mediates glucagon release differently as compared with OCT in humans. Because SOM230 administration did not result in a dramatic attenuation of absolute insulin concentrations, we hypothesize a potential local role of SOM230 in regulating glucose homeostasis in insulin-targeted tissues. Bousquet and coworkers elegantly demonstrated in CHO-K1

cells, stably expressing sst_2, that addition of the sst_2-preferential analog RC-160 to insulin resulted in a higher and more sustained increase of the tyrosine phosphatase SHP-1 association to the insulin receptor (IR) directly correlated with an inhibition of phosphorylation of IR and insulin receptor substrate 1 (IRS-1) (44). Also, activation of sst_1 subtype enhances PTP activity as well (45). The expression of sst_1 and sst_2 in target tissues of insulin action, might point towards potential modulatory effects by SOM230 on insulin signaling. It should also be stated that our skeletal muscle mRNA data were derived from critically ill patients, and we cannot rule out any sst mRNA expression alteration due to the pathological state of these patients (15). SOM230, via sst_1 activation, might increase PTP activation, which subsequently could result in IR- or IRS-dephosphorlyation; plasma glucose levels might remain elevated due to impaired glucose metabolism in peripheral target tissues. It should be noted that OCT administration did not result in a glucose peak 1 h post-injection, whereas the elevated glucose levels around lunch can be explained by inhibition on insulin secretion. Based on sst_1 and sst_2 binding affinity differences between SOM230 and OCT, i.e. a 30-fold higher and 2.5-fold lower affinity, respectively, in combination with the selective expression of sst_1 and sst_2 in liver and adipose tissue, we hypothesize that sst_1 might be involved in SOM230-mediated effects on insulin signaling.

In conclusion, the results show that both dosages SOM230 inhibit free IGF-I more sustained as compared with OCT, which could be regulated centrally and peripherally at the level of the pituitary and liver, respectively. The superior action of OCT compared with SOM230 in stimulating IGFBP-1 levels suggests a modulatory role of sst_2 in the direct regulation of IGFBP-1 levels.. Finally, the acute elevation of glucose levels after SOM230 administration, which cannot be explained by concomitant suppressive effects on insulin concentrations; suggest that extra-pancreatic mechanisms might be involved. The expression of sst_1 and sst_2 in target tissues of insulin action, might point towards modulatory effects by SOM230 on glucose homeostasis. Further *in vivo* and *in vitro* studies with the promising multiligand SRIF-analog SOM230 will be neces-

sary, not only to further explore its potential beneficial role in the medical treatment of sst-positive neuroendocrine tumours, but also to retrieve additional insights regarding its side effects on carbohydrate metabolism.

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Chapter III-4

Somatostatin receptors in peripheral target tissues of insulin action: implications for glucose homeostasis during somatostatin analog treatment?

Abstract

It is well documented that Octreotide (OCT), which is used for the medical treatment of several neuro-endocrine diseases including acromegaly, can suppress insulin secretion by the pancreas and, subsequently, induce a transient raise in glucose level. The novel somatostatin (SRIF) multiligand SOM230, has been demonstrated to have the potential to become a novel therapeutic tool for acromegalic patients, particularly for those patients being partial responsive to OCT. As expected, SOM230 lowered insulin secretion as well but to a much lesser extent compared with OCT. Surprisingly, only SOM230 induced an acute raise in glucose levels, which cannot be explained by insulin suppression. Therefore, it is suggestive that SOM230 alters insulin signaling, i.e. reduces insulin sensitivity. The presence of SRIF receptor subtype (sst) 1 and sst_2 in insulin-target tissues favoured this hypothesis. In a series of visceral omental fat biopsies, we confirm previous findings that only sst_1 and sst_2 mRNA are expressed. In the human hepatoma cell line, Huh-7, sst_1 and sst_2 mRNA was detected as well. When we co-treated Huh-7 cells with insulin, a dose-dependent increase in insulin receptor (IR) autophosphorylation at tyrosine residues 1162 and 1163 was observed. However, neither SOM230 nor OCT was able to alter IR-autophosphorylation, neither in the presence or in the absence of insulin. Whether SRIF-analogs can influence insulin sensitivity more downstream of the IR, i.e. at the level of the insulin receptor substrates, remains to be studied in more detail.

Introduction

The novel multiligand somatostatin (SRIF) analog SOM230 has recently been demonstrated to have the potential to increase the number of acromegalic patients can be biochemically controlled during long-term medical treatment. SOM230, compared with the current clinically available SRIF-analog OCT, can lower circulating growth hormone (GH) concentrations effectively via SRIF receptor subtype (sst) 2 and sst_5, while OCT (at physiological concentrations) can only mediate GH suppression in vivo via sst_2. However, as SRIF and SRIF-analogs modulate the secretion of insulin and glucagon via the sst subtype expression within the endocrine pancreas, a reduced glucose tolerance and even overt hyperglycaemia were initially expected during long-term therapy of acromegaly. In general, the effects

of SRIF-analogs on glucose homeostasis appeared to be minor and a mild deterioration occurred only in those without impaired glucose tolerance (1). Indeed, compared with OCT, comparable post-prandial elevations of glucose concentrations after lunch were observed after SOM230 administration (2). In addition, 250µg SOM230 elicited an acute rise of glucose levels 1 h post-injection (3). SOM230-mediated inhibitory effects on insulin secretion cannot explain these elevated glucose levels. While glucagon release appeared far less sensitive for SOM230 as compared with OCT (44-fold) in several animal models in vivo, and because peripheral glucagon measurement does not represent pancreatic glucagon (gastrointestinal glucagon is measured as well) concentrations, the effects of SOM230 and OCT on glucagon levels were not analyzed. Since SOM230 administration did not result in a dramatic attenuation of absolute insulin concentrations, we hypothesize a potential local role of SOM230 in regulating glucose homeostasis in insulin-targeted tissues. The expression of only sst_1 and sst_2 in target tissues of insulin action (3), might point towards potential modulatory effects by SOM230 on insulin sensitivity. Therefore, we carried out additional experiments in a series of visceral omental fat tissues as well as in Huh-7 cells, to further explore whether sst expression and activation can alter the insulin receptor signaling cascade.

Methods

Patients and RNA isolation

Sst mRNA expression in visceral (omental) fat tissue was analyzed. Peroperative obtained human omental fat biopsies from 6 patients were collected. All patients underwent a Whipple procedure because of chronic fibrosing pancreatitis (n=1), Vater's papilla carcinoma (n=2) or pancreas carcinoma (n=3). The collected omental tissues were directly snap frozen and stored at -80 °C. The protocols were in accordance with the Helsinki Doctrine on Human Experimentation, and were performed according to the rules of the hospital medical ethical committee. Informed consent was obtained from all patients. Collected tissues were grinded to powder on dry ice using a mortar and total RNA was isolated using RNeasy®

Lipid Tissue Mini Kit for adipose tissue samples (QIAGEN, Westburg B.V., Leusden, The Netherlands) according to manufacturers protocol.

Cell culture

Huh-7 cells, a human hepatoma cell line, were routinely passaged by trypsinization as described in detail previously (4). The cells were maintained in 75 cm^2 flasks in Dulbecco's Minimal Essential Medium (DMEM), supplemented with non essential amino acids, sodium pyruvate (1 mmol/L), 10% fetal calf serum (FCS), penicillin ($1x10^5$ U/L), fungizone (0.5 mg/L), L-glutamine (2 mmol/L), and sodium bicarbonate (2.2 g/L), pH 7.6. The cells were cultured at 37° C in a CO_2-incubator. Media and supplements were obtained from GIBCO Bio-cult Europe (Invitrogen, Breda, The Netherlands).

Quantitative PCR

Quantitative PCR was performed as described previously (5). Messenger RNA was isolated using Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway) from 1.0 X 10^6 cells Huh-7 cells or isolated visceral omental adipocyte tissue. The cells were lysed for 2 min in an ice-cold Tris-buffer (100 mM Tris-HCl, pH 8, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM DTT and 5 U/100µl RNAse inhibitor (HT Biotechnology Ltd., Cambridge, UK). The mixture was centrifuged at 14,000 rpm for 1 min to remove cell debris. After adding 40 µl pre-washed Dynabeads Oligo $(dT)_{25}$ to the supernatant, the mixture was incubated for 5 min on ice. Thereafter, the beads were collected with a magnet, washed three times with a Tris-buffer (10 mM Tris HCl, pH 8, 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS), and once with a similar buffer from which LiDS was omitted. Messenger RNA was eluted from the beads in 2 x 20 µl H_2O for 2 min at 65 °C. Complementary DNA (cDNA) was synthesized using the poly A^+ mRNA in a Tris-buffer (50 mMTris-HCl, pH 8.3, 100 mM KCl, 4 mM DTT, 10 mM $MgCl_2$) together with 1 mM of each deoxynucleotide triphosphate, 10 U RNAse inhibitor, and 2 U AMV Super Reverse Transcriptase (HT Biotechnology Ltd., Cambridge, UK) in a final volume of 40 µl. This mixture was incubated for 1 h at 42 °C. One tenth of the cDNA library was used

for quantification of sst subtype mRNA levels. The assay was performed using 15 µl TaqMan Universal PCR master mix (Applied Biosystems, Capelle aan de IIssel, The Netherlands), 500 nM forward primer, 500 nM reverse primer, 100 nM probe and 10 µl cDNA template, in a total reaction volume of 25 µl. The reactions were carried out in a ABI 7700 sequence detector (The Perkin-Elmer Corporation, Foster City, CA). PCR amplification started with a first step for 2 min at 50 °C, followed by an initial heating at 95 °C for 10 min and, subsequently, samples were subjected to 40 cycles of denaturation at 95 °C for 15 sec and annealing for 1 min at 60 °C. To ascertain that no detectable genomic DNA was present in the poly A^+ mRNA preparation, since sst genes are intron-less, the cDNA reactions were also performed without reverse transcriptase. The detection of hypoxanthine-phosphoribosyl-transferase (hprt) mRNA served as a control and was used for normalization of the sst subtype mRNA levels. The primer sequences that were used have been described in detail previously (6). In addition, the relative amount of sst subtype mRNA was determined using a standard curve generated from known amounts of human genomic DNA. For determination of the amount of hprt mRNA, a standard curve was generated of a pool of cDNAs from a human cell line known to express hprt (6). The relative amount of sst subtype mRNA was calculated by normalization to the amount of hprt mRNA and is given in arbitrary units. Each sample was assayed in duplicate.

Insulin receptor (IR) autophosphorylation assay

After trypsinizing confluent 75 cm^2 flasks, Huh-7 cells were dispersed in 6 well plates (500.000 cells/well). The following day, culture medium was removed and cells were maintained for 1 hr in Krebs buffer (pH 7.4). Subsequently, Krebs buffer was refreshed and Huh-7 cells were incubated with different concentrations of insulin (10nM-1pM) and 10nM SOM230 or OCT (as indicated in *Figure legends*). Washing the cells with ice-cold PBS terminated incubation. Huh-7 were collected in PBS and centrifuged. Supernatant was removed and the cell pellet lysed for 30 minutes, on ice, in 1mL of Cell Extraction Buffer [10mM Tris, pH 7.4;

100mM NaCl; 1mM EDTA; 1mM EGTA; 1mM NaF; 20mM $Na_4P_2O_7$; 2mM Na_3VO_4; 1% Triton X-100; 10% glycerol; 0.1% SDS; 0.5% deoxycholate; 1mM PMSF and Protease inhibitor cocktail (Sigma, cat. no P-2714, 250µl per 5mL cell extraction buffer)]. Cell extract was centrifuged for 10 min at 4°C at 13,000 rpm and clear lysates were stored at -80°C. 100µl clear lysate was diluted 10-fold and used, according to the manufacturer's protocol, in a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA), designed to detect and quantify the levels of IR that are phosphorylated at tyrosine (Tyr) residues 1162 and 1163 of IR (Biosource, Nivelles, Belgium). Briefly, during the first 2 hr incubation the IR antigen binds to a monoclonal antibody specific for IR (β-subunit), which has been coated on the wells. After washing, an antibody specific for IR phosphorylated at Tyr1162 and Tyr1163 is added to the wells for 1 hr. After removal of excess detection antibody, a horseradish peroxidase (HRP)-labeled anti-rabbit IgG is added for 30 min, completing the four-member sandwich. After washing to remove excess anti-rabbit IgG-HRP, a substrate solution was added for 30 min in the dark, which is acted upon the bound enzyme to produce colour. After addition of a stop solution, wells were stirred and absorbance (optical density) was detected at 450 nm. The intensity of the colored product is directly proportional to the concentration of IR [pYpY1162/1163] present in the original specimen. 1 Unit of standard is equivalent to the amount of IR [pYpY1162/1163] derived from 0.6 ng of IR in CHO cells, transfected with human IR, stimulated with 100 nM insulin. The intra- and inter-assay coefficients of variation were 4.85% and 5.98%, respectively.

Statistical analysis

The statistical significance of the difference between the effects of 10nM-1pM insulin and 10 nM SRIF-analogs in the IR-autophosphorylation ELISA assay was determined by using one-way analysis of variance (ANOVA). When significant overall effects were obtained by this method, comparisons were made using Newman-Keuls multiple comparisons test. Data are reported as means ± SEM of the indicated n values, unless otherwise specified.

Results

Sst mRNA expression

In all six visceral omental adipocyte tissues, both sst_1 and sst_2 were expressed (Fig. 1A: relative copy number 435 ± 103 and 841 ± 151, respectively, $p<0.01$). The other sst subtypes, sst_{3-5}, were not expressed. As depicted in Figure 1B, Huh-7 cells expressed sst_1 (relative copy number 201 ± 22) and sst_2 (relative copy number 357 ± 44; $p<0.01$ vs sst_1), while sst_{3-5} mRNA expression was not found.

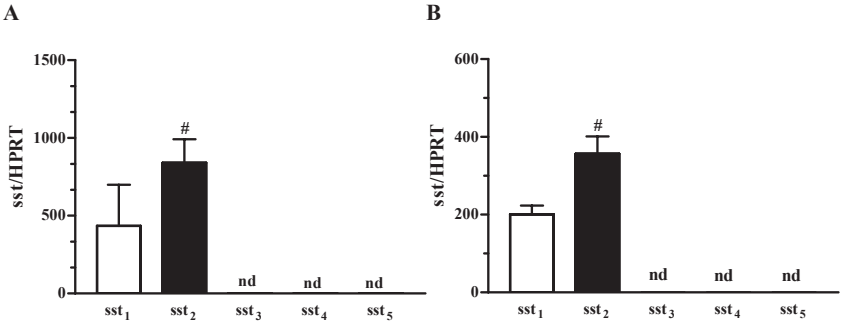

Figure 1. Quantitative analysis of RT-PCR showing the relative amounts of the five SRIF receptor subtypes, sst_{1-5}, mRNA in visceral omental fat tissue (A) and Huh-7 cells (B), calculated relative to the amount of HPRT and given in arbitrary units. A) Data represent the mean ± S.E. of 6 visceral fat specimens, of which each individual patient sample was assayed in duplicate. B) Data represent the mean ± S.E. of three independent experiments, performed in duplicate. nd; not detectable. #, $p<0.01$ vs sst_1.

IR-autophosphorylation in Huh-7 cells

First, we explored the insulin-induced concentration-dependent autophosphorylation of the IR in Huh-7 cells during different incubation periods, *i.e.* 2, 5 and 30 minutes. After all three incubation periods, a dose-dependent increase of IR-autophosphorylation by insulin was observed (Fig. 2). After 2 minutes of insulin treatment, depicted in figure 2A, a significant 3 to 4-fold increase in IR-autophosphorylation in Huh-7 cells by 10 and 1 nM insulin was observed. In addition, after 5 minutes of insulin treatment (Fig. 2B), 0.1 and 0.01 nM insulin also significantly increased IR-autophosphorylation in Huh-7 cells compared with non-stimulated cells. At 30 minutes of insulin treatment, the increase in IR autophosphorylation was reduced compared with the 2 and 5 minutes

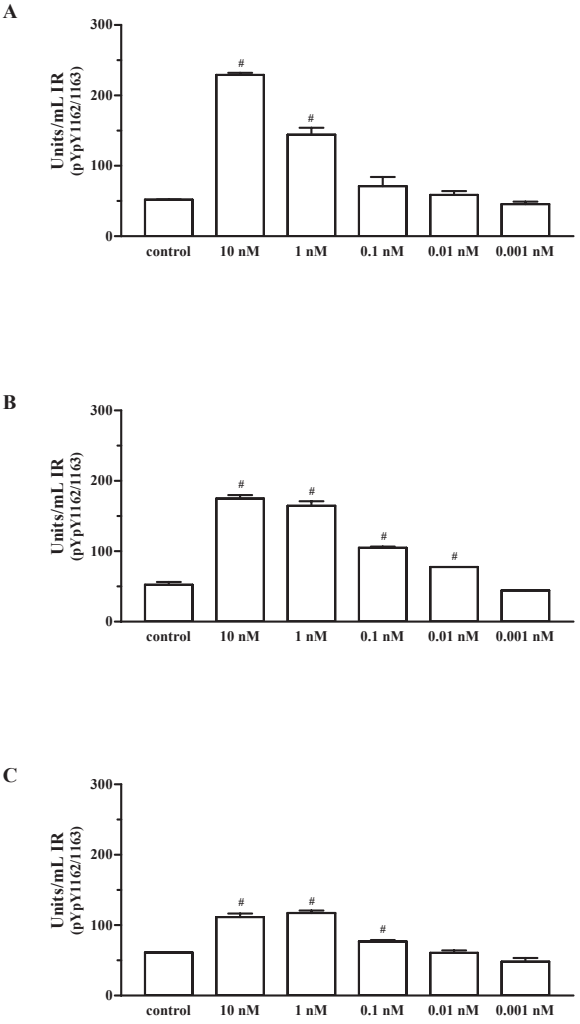

Figure 2. Concentration-dependent induction of IR-autophosphorylation by insulin. Huh-7 cells were dispersed in 6-wells plates, treated for 2 (A), 5 (B) and 30 (C) minutes with insulin (10 nM – 0.001 nM range) in Krebs buffer (ph 7.4). A solid phase sandwich ELISA, was used to detect and quantify the levels of IR that are phosphorylated at tyrosine residues 1162 and 1163 of IR in the lysates (Biosource, Nivelles, Belgium). Data are expressed as Units/mL IR, and represent the mean ± S.E. of separate experiments, performed in duplicate. #, $p<0.01$ vs. non-stimulated Huh-7 cells (control). When error bars are not apparent the S.E. values were smaller than symbol size.

incubation periods, although a 2-fold increase by 10 and 1nM insulin compared with non-stimulated Huh-7 cells was still observed (Fig. 3C). On the basis of these 'control' experiments, we chose a 3-minute incuba

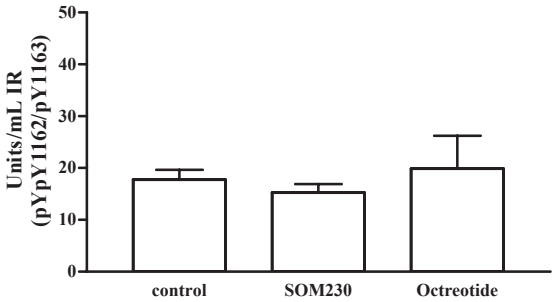

Figure 3. Effect of SOM230 and OCT on IR-autophosphorylation. Huh-7 cells were dispersed in 6-wells plates, treated for 3 minutes with 10 nM SOM230 or 10 nM OCT in Krebs buffer (pH 7.4). Data are expressed as Units/mL IR, and represent the mean ± S.E of two individual experiments, performed in duplicate.

tion period with physiological concentrations of 1 and 0.1 nM insulin, to evaluate whether co-treatment with 10 nM SOM230 or OCT can alter insulin-induced IR-autophosphorylation in sst_1+sst_2 expressing Huh-7 cells. The incubation of Huh-7 cells with either 10 nM SOM230 or 10 nM OCT did not induce any change in IR-autophosphorylation (Fig. 3).

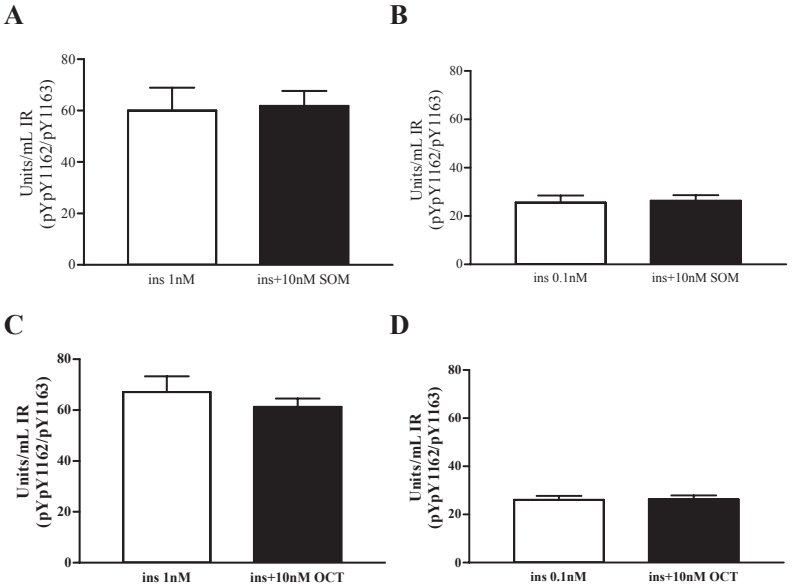

Figure 4. Effect of SOM230 and OCT on insulin-induced IR-autophosphorylation. Huh-7 cells were dispersed in 6-wells plates, treated for 3 minutes with insulin in the presence or absence of a SRIF-analog in Krebs buffer (pH 7.4). (A) 1nM insulin +/- 10 nM SOM230, (B) 0.1nM insulin +/- 10 nM SOM230, (C) 1nM insulin +/- 10 nM OCT and (D) 0.1nM insulin +/- 10nM OCT. Data are expressed as Units/mL IR, and represent the mean ± S.E of 2 separate experiments, performed in triplicate. □, insulin; ■, insulin + SRIF-analog.

When Huh-7 cells were treated for 3 minutes with 1 and 0.1 nM insulin either alone or in combination with 10 nM SOM30, IR-autophosphorylation was not affected (Fig. 4A+B). As depicted in figure 4C and 4D, comparable results as for SOM230 were observed when Huh-7 cells were co-treated with insulin (1 and 0.1 nM) and 10 nM OCT.

Discussion

SOM230 administration in acromegalic patients does not result in a dramatic attenuation of absolute insulin concentrations that can totally explain the acute rise in glucose levels (3). Therefore, we hypothesized that SOM230 might induce altered insulin signaling in major target tissues such as liver, skeletal muscle and adipose tissue, *i.e.* the acute s.c administration of SOM230 in acromegaly seems to attenuate insulin sensitivity. During a single-dose Phase I study with SOM230, the increase in serum glucose levels was enhanced post-prandially as well and appeared dose-dependent, while similar observations were reported in multiple-dose studies (7). Crucial for a potential role of SRIF-analogs, in particular SOM230, in modulating the insulin signaling cascade, is the presence of sst in these insulin-target tissues. The sst mRNA expression pattern observed in all individual 6 specimens from visceral omental fat tissue, i.e. only sst_1 and sst_2 mRNA, are in agreement with previously reported mRNA data in visceral fat tissue (3). The insulin receptor (IR) is a tetrameric glycoprotein consisting of two α and two β subunits linked by disulfide bonds. The intracellular β subunit is a tyrosine kinase that is activated when insulin binds to the extracellular α-subunit. The tyrosine kinase autophosphorylates the insulin receptor and initiates subsequent intracellular phosphorylations that mediate the multiple actions of insulin (8). The predominant downstream signal after IR autophosphorylation is activation of the insulin receptor substrate (IRS) proteins (9). IRS-1 and other recently cloned IRS proteins (IRS-2, -3, -4) are phosphorylated upon insulin stimulation and have adaptor functions between the IR and other cellular substrates (10). Insulin increases glucose uptake in muscle and fat, inhibits hepatic glucose production and promotes the storage of substrates in fat, liver and muscle, and inhibits lipolysis, glycogenolysis and protein breakdown (11).

Insulin action can be attenuated by protein tyrosine phosphatases (PTP), which catalyse the rapid dephosphorylation of the receptor and the IRS substrates (8, 12). The five SRIF receptors are known to mediate a variety of signal transduction pathways, including protein dephosphorylation through PTP activation (13-15). Several *in vitro* studies using sst-expressing tumor cells already demonstrated that SRIF-receptors can activate tyrosine phosphatases, thereby slowing tumor cell growth stimulated by tyrosine kinases (16-18). In particular, Bousquet and coworkers elegantly demonstrated in CHO-K1 cells, stably expressing sst_2, that addition of the sst_2-preferential analog RC-160 to insulin resulted in a higher and more sustained increase of the tyrosine phosphatase SHP-1 association to IR, that was directly correlated with an inhibition of phosphorylation of IR and IRS-1 (19). Also, activation of sst_1 subtype enhances PTP activity as well (20). The expression of sst_1 and sst_2 in target tissues of insulin action, might form the molecular target point for effects by SOM230 on insulin signaling. SOM230, via sst_1 and/or sst_2 activation, might increase PTP

Table I. Binding selectivity of SRIF-analogs for the five sst subtypes.

Compound	Binding affinity (IC_{50}, nM)				
	sst_1	sst_2	sst_3	sst_4	sst_5
Somatostatin-14	2.3	0.2	1.4	1.8	1.4
Octreotide	280	0.4	7.1	>1000	6.3
SOM230	9.3	1.0	1.5	>100	0.2

Data are from radioligand binding assays to membranes from transfected CHO-K1 cells and African green monkey kidney cells expressing the different human sst subtypes (28).

activation, which subsequently could result in IR- or IRS-dephosphorylation. Eventually, by attenuating the insulin-signaling cascade, plasma glucose levels might remain elevated due to impaired glucose metabolism in peripheral target tissues. Based on sst_1 and sst_2 binding affinity

differences between SOM230 and OCT, i.e. a 30-fold higher and 2.5-fold lower affinity, respectively (Table I), we hypothesize that sst_1 alone, or in combination with sst_2, might be involved in SOM230-mediated effects on insulin signaling. This would also explain why OCT 100μg did not induce a profound glucose release, because this OCT dosage is by far not enough to activate sst_1 due to its very low sst_1 binding affinity (EC_{50} >100 nM). However, our experiments in Huh-7 cells, which were shown to express only sst_1 and sst_2 at the mRNA level which, do not demonstrate that sst_1 or sst_2 activation can attenuate tyrosine phosphorylation at the IR itself. We selected Tyr1162and Tyr1163, since the catalytic loop of the tyrosine kinase domains of the IR involves a three-tyrosine motif corresponding to Tyr1158, 1162 and 1163 (21). It is generally believed that autophosphorylation within the activation loop proceeds a progressive manner initiating at the second tyrosine (1162), followed by phosphorylation at the first tyrosine (1158), then the last (1163), upon which the IR becomes fully active (21). The 10 nM concentration of both SRIF-analogs should have been sufficiently high to activate sst_1 (only by SOM230) or even sst_2. Possibly, attenuation of insulin signaling by SOM230 could also take place at the level of IRS-1or IRS-2 proteins. Both IRS-1 and IRS-2 knockout mice exhibit insulin resistance (22-25) while IRS-3 and IRS-4 knockout mice have a normal metabolic profile (26). Therefore, it will be interesting to evaluate whether phosphorylation of IRS-1 and/or IRS-2 can be altered via sst_1 and/or sst_2 activation by SOM230. It should also be notified that the Huh-7 cells have not been thoroughly screened regarding their IR-pharmacology; other human hepatoma cell lines or, preferentially, human primary cell cultures should be investigated as well to evaluate the IR-autophosphorylation involvedness in more detail. Moreover, human primary adipocytes and myocytes have to be investigated as well, since these two represent the other target organs of peripheral insulin action throughout the human body. *In vivo* analysis of IR-autophosphorylation in mice can also form a potential target to investigate the role of sst in insulin signaling, although interspecies variation can be a burden for translating data from mice to men. Still, mice lacking PTP1B are hyperresponsive to insulin, and liver specific re-expression of PTP1B in these

PTP1B knock-out mice led to marked attenuation of their enhanced insulin sensitivity (27). This observation was probably caused by preferential dephosphorylation of Tyr1162/1163 residues of the IR by PTP1B *in vivo*.

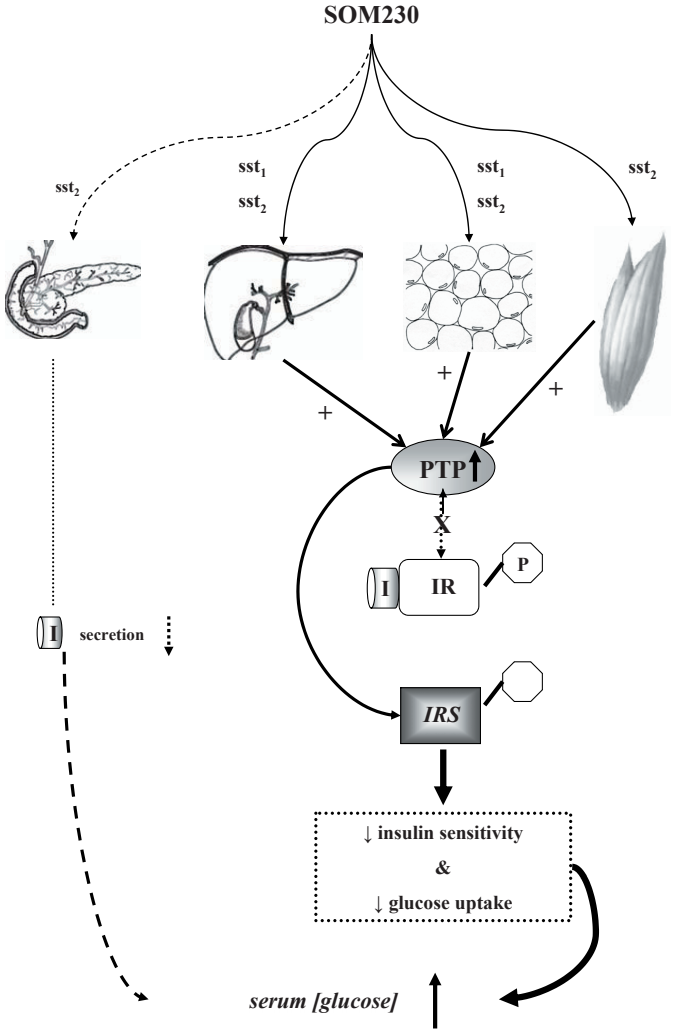

Figure 5. Schematic overview of possible physiological pathways involved in the hyperglycaemic effect induced by SOM230. I, Insulin; IR, Insulin receptor; IRS, Insulin receptor substrate; P, phosphorylated state of the receptor/protein; PTP, protein tyrosine phosphatase and sst, SRIF-receptor subtype. Solid lines represent the proposed primary metabolic route of SOM230-induced hyperglycaemia via alteration of insulin sensitivity in peripheral target organs of insulin action; dashed lines form the minor involvement of SOM230-mediated suppression of insulin secretion.

In figure 5, we have summarized our data with respect to the potential physiological pathways involved in the hyperglycaemic effect induced by SOM230. Our data in 12 acromegalic patients demonstrated that SOM230 lowers insulin secretion from pancreatic β-cells. The inhibition of insulin secretion seems to be mediated via sst_2 while OCT, with a 2.5 fold higher sst_2 binding affinity as SOM230, inhibited insulin secretion more pronounced as compared with SOM230. In addition, only SOM230 injection resulted in an acute raise in glucose levels. Therefore, we hypothesized that SOM230 might alter insulin signaling, i.e. SOM230 lowers insulin sensitivity. The sst_1 and sst_2 mRNA expression in peripheral target tissues of insulin action, namely liver, visceral fat and muscle, form a crucial factor that might point towards a role of sst in the insulin signaling cascade. Based on the binding affinities of SOM230 and OCT for sst_1 and sst_2, and the observation that only SOM230 caused an acute raise in glucose levels, we suggest that sst_1 alone or in combination with sst_2, might be responsible for attenuating insulin signaling. SOM230, may activate PTPs, leading to dephosphorylation of IRS proteins but not the IR, as no alteration of the IR-autophosphorylation by SOM230 or OCT in Huh-7 cells was observed. Eventually, this would increase insulin resistance, which, together with a subtle suppression of insulin secretion, results in elevated serum glucose levels.

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Chapter IV

Functional Characterisation of Somatostatin Receptor Subtypes 2 and 5

Chapter IV-1

Functional interplay between somatostatin receptor subtype 2 and 5 when variably co-expressed in vitro

submitted

Abstract

Although recent insights demonstrate that somatostatin (SRIF) receptor subtype (sst) 5, compared with sst_2, is highly expressed in the majority of growth hormone (GH-) secreting pituitary adenomas, it has been suggested that the sst_2 is the (pre-) dominant receptor in regulating GH release by GH-secreting pituitary adenoma cells *in vivo* and *in vitro*, whereas sst_5 receptors may mediate an inhibitory effect on GH secretion as well. The objective of the present study was to investigate whether a functional interplay occurs between sst_2 and sst_5. Therefore, a cAMP Response Element-Luciferase Reporter Gene Assay and [^{125}I-Tyr^{11}]-SRIF-14 radioligand binding studies were used in several series of transiently co-transfected HEK 293 cells expressing a constant level of sst_2 mRNA and sst_5 mRNA varying from low (sst_2>>sst_5) to high (sst_2<<sst_5). The efficacy of the sst_2-preferring SRIF-analog Octreotide (OCT) appeared not to be affected by the different sst_2/sst_5 expression ratios, whereas the sst_5-preferring SRIF-analog BIM-23206 was only maximally effective if the sst_5 expression level was superior to sst_2 expression. The same pattern as for BIM-23206, although to a lesser extent was observed for the multiligand SOM230. In conclusion, these results demonstrate a functional interplay between the key-players in the regulation of human GH release. The sst_2 appears to function in a dominant manner over sst_5, while sst_5 itself can only serve at maximal potency when sst_5 expression far exceeds that of sst_2.

Introduction

Somatostatin (SRIF) exerts its biological effects via five distinct high affinity membrane receptor subtypes (sst) that belong to the family of G-protein-coupled receptors (GPCR) (1). These sst are particularly densely and homogenously distributed over the growth hormone (GH-) secreting pituitary tumors of acromegalic patients (2-4). Most GH-secreting pituitary adenomas predominantly express mRNA and protein for sst_2 and sst_5, while sst_1 and sst_3 are moderately expressed and sst_4 are not found (5-7). SRIF binds with high affinity to all five sst subtypes (8), whereas currently available SRIF-analogs octreotide (OCT) and lanreotide display a high, moderate and low affinity to sst_2, sst_{3+5} and sst_{1+4}, respectively (9). The sst_2-preferring SRIF analogs have been demonstrated to control hormonal hypersecretion successfully in about two-thirds of acromegalic patients

(10-12). In acromegalic patients only partially responsive to OCT or lanreotide, a relatively low expression of sst_2 receptors in the GH-secreting pituitary adenomas of these patients likely explains the partial sensitivity to these SRIF-analogs (13). Recent insights show that sst_5 receptors are highly expressed in the majority of GH-secreting pituitary adenomas (13, 14). Novel SRIF-analogs targeting both sst_2 and sst_5 seem more potent in suppressing GH release, compared with sst_2 selective SRIF-analogs (15). A first proof-of-concept clinical trial testing a single s.c. administration of SOM230, a SRIF-analog with a more universal binding to sst (16, 17), showed a significant suppression of circulating GH levels in 11 of 12 patients, suggesting that novel SRIF-analogs with binding affinity to both sst_2 and sst_5 have the potency the increase the number of patients that can be controlled biochemically during long-term medical treatment (18). GH inhibition from GH-secreting pituitary adenomas by OCT correlates, *in vitro* as well as *in vivo*, with quantitative expression of sst_2 mRNA but not for sst_5 mRNA (13, 14). Surprisingly, although SOM230 shows a very high binding affinity for sst_5 receptors, no significant correlation was found between the *in vitro* GH suppression by a maximally active concentration of SOM230 and sst_5 mRNA levels, whereas a positive correlation was found between the effects of SOM230 and sst_2 mRNA levels (14). Therefore, it has been suggested that the sst_2 seems the (pre-) dominant receptor in regulating GH release by GH-secreting pituitary adenoma cells, whereas sst_5 receptors may mediate an inhibitory effect on GH secretion as well. A direct proof for this hypothesis is currently lacking, however. Therefore, the objective of the present study was to use different sst subtype selective and universal SRIF-analogs in HEK293 cells, transfected with variable quantities of human sst_2 and sst_5 receptors to investigate whether a functional interplay occurs between sst_2 and sst_5.

Materials and Methods

Cell line culture and sst constructs

HEK 293 cells (kind gift of Dr. A.P.N. Themmen, Internal Medicine, Erasmus MC Rotterdam, the Netherlands) were routinely passaged by

trypsinization as described in detail previously (19). The cells were maintained in 75 cm^2 flasks in DMEM/F-12 medium, supplemented with non essential amino acids, sodium pyruvate (1 mmol/L), 10% fetal calf serum (FCS), penicillin ($1*10^5$ U/L), streptomycin (50 mg/L), fungizone (0.25 mg/L), L-glutamine (2 mmol/L), and sodium bicarbonate (2.2 g/L), pH 7.6. The cells were cultured at 37° C in a CO_2-incubator. Before transfection, the cells were seeded at 40% confluence in 75 cm^2 flasks and transfected the next day using the calcium phosphate precipitation method. In order to create a series of different sst_2/sst_5 ratios being expressed, HEK 293 cells were transiently transfected with various concentrations of human sst_2 and sst_5 cDNA [human sst_2 or sst_5 cDNA in pBluescript (pBS) (a kind gift of G.I. Bell, Howard Hughes Medical Institute Chicago, Illinois) was excised from pBS and inserted into the Nhe-1/Sal1 or EcoRI/XbaI cloning site, respectively, of the mammalian expression vector pCi-neo (Promega Benelux, Leiden, the Netherlands)]. Media and supplements were obtained from GIBCO Bio-cult Europe (Invitrogen, Breda, the Netherlands).

Forskolin-induced cAMP Response Element-Luciferase Reporter Gene Assay

The functional responses of the SRIF-analogs in the various cell systems were determined using a cAMP-responsive reporter construct that contains six cAMP response elements in tandem in front of the cDNA encoding the luciferase (LUC) reporter enzyme [pCRE6lux (20)]. HEK293 cells were co-transfected with pCRE6Lux (20) and pRSVlacZ, to control for transfection efficiency (21) (maximum of 10 µg sst-expression construct, 2 µg pRSVlacZ, 2 µg pCRE6Lux, and 6 µg carrier DNA per ml precipitate). Three days after transfection the SRIF-analog dependent CRE-LUC response was determined in 48-well tissue culture plates (Costar, Cambridge, MA) by incubating the cells for 6 h in culture medium containing 0.1% BSA with 1 µM forskolin (FSK) and increasing concentrations of SRIF-analog (range 100 nM – 0.01 pM) and, in another series of experiments, also in the absence or presence of 50 nM BIM-23454 (sst_2-antagonist). Subsequently, the media were aspirated, the cells

lysed and luciferase activity was measured using a TopCount luminometer after adding luciferin (22). β-Galactosidase activity of the lysates was determined to correct for transfection efficiency (21). The CRE-LUC response produced by 1 μM FSK in each experiment was set at 100%.

Radioligand binding studies

Scatchard Analysis

To determine the binding affinity (Kd) and total receptor number (Bmax), HEK293 cells were transfected with different amounts of sst_2 and sst_5 DNA (maximum of 14μg total sst-expression construct and 6 μg carrier DNA per ml precipitate). Three days after transfection, Scatchard analysis using [^{125}I-Tyr^{11}]-SRIF-14 binding experiments was performed. The method of membrane isolation and the reaction conditions were previously described (23). Briefly, membrane preparations (corresponding to 50 μg protein) of transiently transfected HEK 293 cells, were incubated in a total volume of 100 μl at room temperature for 45 min with increasing concentrations of [^{125}I-Tyr^{11}]-SRIF-14 and with or without excess (1 μM) of unlabeled Tyr^{11}-SRIF-14, respectively, in Hepes buffer (10 mM Hepes, 5 mM $MgCl_2$ and 0.02 g/L bacitracin, pH 7.6) containing 0.2% BSA. After the incubation, 1 mL ice-cold Hepes buffer was added to the reaction mixture, and membrane-bound radioactivity was separated from unbound by centrifugation during 2 min at 14,000 rpm in a Eppendorf microcentrifuge. The remaining pellet was washed twice in ice-cold Hepes buffer, and the final pellet was counted in a γ-counter. Specific binding was taken to be total binding minus binding in the presence of 1 μM unlabeled Tyr^{11}-SRIF-14.

Competition experiments

In competition experiments, membrane preparations of transiently transfected HEK 293 cells, as described above, were incubated in a total volume of 100 μl at room temperature for 45 min with 25 μl [^{125}I-Tyr^{11}]-SRIF-14 (40.000cpm) and either 25 μl binding assay buffer (total binding) or 1 nM OCT, SOM230, BIM-23206 or OCT and BIM-23206, in Hepes buffer (10 mM Hepes, 5 mM $MgCl_2$ and 0.02 g/L bacitracin, pH 7.6) containing 0.2%

BSA. After the incubation, 1 mL ice-cold Hepes buffer was added to the reaction mixture, and membrane-bound radioactivity was separated from unbound by centrifugation during 2 min at 14,000 rpm in an Eppendorf microcentrifuge. The remaining pellet was washed twice in ice-cold Hepes buffer, and the final pellet was counted in a γ-counter. Experiments were conducted in triplicate for each separate co-transfection of sst_2 and sst_5.

Quantitative PCR

Quantitative PCR was performed as described previously (24). Messenger RNA was isolated using Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway) from transiently transfected (72 h) HEK 293 cells. The cells were lysed for 2 min in an ice-cold Tris-buffer (100 mM Tris-HCl, pH 8, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM DTT and 5 U/100µl RNAse inhibitor (HT Biotechnology Ltd., Cambridge, UK). The mixture was centrifuged at 14,000 rpm for 1 min to remove cell debris. After adding 40 µl pre-washed Dynabeads Oligo $(dT)_{25}$ to the supernatant, the mixture was incubated for 5 min on ice. Thereafter, the beads were collected with a magnet, washed three times with a Tris-buffer (10 mM Tris HCl, pH 8, 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS), and once with a similar buffer from which LiDS was omitted. Messenger RNA was eluted from the beads in 2 x 20 µl H_2O for 2 min at 65 °C. Complementary DNA (cDNA) was synthesized using the poly A^+ mRNA in a Tris-buffer (50 mMTris-HCl, pH 8.3, 100 mM KCl, 4 mM DTT, 10 mM $MgCl_2$) together with 1 mM of each deoxynucleotide triphosphate, 10 U RNAse inhibitor, and 2 U AMV Super Reverse Transcriptase (HT Biotechnology Ltd., Cambridge, UK) in a final volume of 40 µl. This mixture was incubated for 1 h at 42 °C. One tenth of the cDNA library was used for quantification of sst subtype mRNA levels. The assay was performed using 15 µl TaqMan Universal PCR master mix (Applied Biosystems, Capelle aan de IJssel, The Netherlands), 500 nM forward primer, 500 nM reverse primer, 100 nM probe and 10 µl cDNA template, in a total reaction volume of 25 µl. The reactions were carried out in a ABI 7700 sequence detector (The Perkin-Elmer Corporation, Groningen, The Netherlands). PCR amplification started with a first step for 2 min at 50 °C, followed by an initial

heating at 95 °C for 10 min and, subsequently, samples were subjected to 40 cycles of denaturation at 95 °C for 15 sec and annealing for 1 min at 60 °C. To ascertain that no detectable genomic DNA was present in the poly A^+ mRNA preparation, since sst genes are intron-less, the cDNA reactions were also performed without reverse transcriptase. The detection of hypoxanthine-phosphoribosyl-transferase (hprt) mRNA served as a control and was used for normalization of the sst subtype mRNA levels. The primer sequences that were used have been described in detail previously (14).

Dopamine D2 receptor(D2R) assay

In order to create both D2R-expressing as well D2R and sst_2+sst_5 co-expressing HEK 293 cells, transient transfections were carried out with human D2R cDNA (commercially available at UMR cDNA resource center; www.cDNA.com) in the absence and presence of human sst_2+sst_5 cDNA, respectively. Quantitative RT-PCR for the detection of D2R mRNA expression was carried out as above, whereby the concentration of forward primer, reverse primer and probe were 300 nM, 300 nM and 200 nM, respectively. The D2R-primer sequences that were used were;
Forward: 5'- GCCACTCAGATGCTCGCC-3,
Reverse: 5'- ATGTGTGTGATGAAGAAGGGCA-3' and
Probe: 5 'FAM - TTGTTCTCGGCGTGTTCATCATCTGC-TAMRA-3

Test-substances

Octreotide (OCT, Sandostatin®) was obtained from Novartis Pharma A.G., (Basel, Switzerland). SOM230 was synthesized by Novartis Pharma A.G. Somatostatin-14 was purchased from Sigma Chemical Co. (St. Louis, MO). BIM-23206, a sst_5-subtype specific analog, and BIM-23454, a sst_2 antagonist, were synthesized by IPSEN (Massachusetts, USA). Sst binding affinities are depicted in Table I. [^{125}I-Tyr^{11}]-SRIF-14 was purchased from Amersham (Houten, The Netherlands). Cabergoline was obtained from Pharmacia-Pfizer (Rome, Italy).

Statistical analysis

The statistical significance of the difference between the effects of 1 nM

SRIF-analog in the CRE-LUC Reporter Gene Assay and radioligand competition experiments were determined by using one-way analysis of variance (ANOVA). When significant overall effects were obtained by this method, comparisons were made using Newman-Keuls multiple comparisons test. Calculation of IC_{50} values for inhibition of FSK-induced cAMP response was made using GraphPad Prism version 3.02 (San Diego, CA). The unpaired Student t-test was chosen to analyze differences in concentration-effect curves. Pearson correlation coefficient was used for correlation analysis between concentration-effect curves and the sst_5 mRNA expression levels (both Log-transformed). Data are reported as means ± SEM of the indicated n values, unless otherwise specified.

Table I. Binding selectivity of SRIF-analogs for the five human sst receptor subtypes used in this study.

Compound	Binding affinity (IC_{50}, nM)				
	sst_1	sst_2	sst_3	sst_4	sst_5
SRIF-14[a]	1.1	0.2	1.4	0.5	1.4
Octreotide	280	0.4	7.1	>1000	6.3
SOM230	9.3	1.0	1.5	>100	0.2
BIM-23206	>1000	166	1000	>1000	2.4
BIM-23454 ≠	ND	31.6	ND	ND	138.7

Data are from radioligand binding assays to membranes from transfected CHO-K1 cells (16, 28) and African green monkey kidney cells (16) expressing the different human sst subtypes. Values are from IPSEN (Culler, M.D.), and from Lewis and coworkers (16). Data for SRIF-14 are summarized from Refs (17, 25-28). ≠, sst2 antagonist; ND, not done.

Results

cAMP response

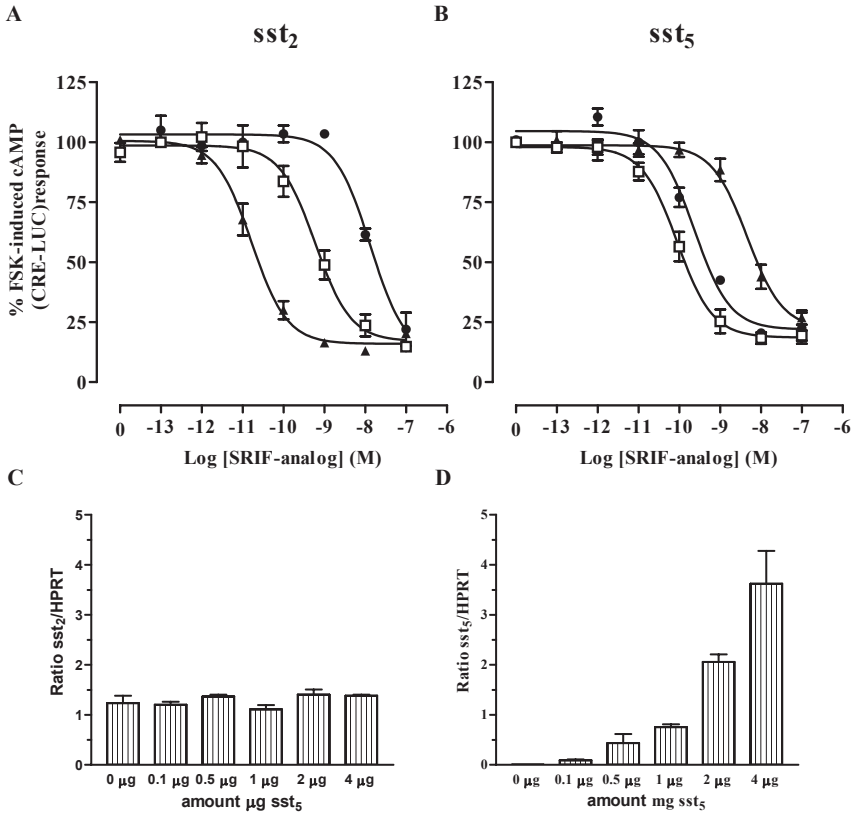

Figure 1. Dose-dependent inhibition of FSK-induced cAMP response by OCT (▲), SOM230 (□) and BIM-23206 (●) in transiently transfected HEK 293 cells, expressing only sst_2 (A) or sst_5 (B). cAMP response was determined with the use of six copies of a cAMP response element (CRE)-luciferase reporter gene construct (CRE-LUC). β-Galactosidase activity of the lysates was determined to correct for transfection efficiency. The cAMP (CRE-LUC) response produced by 1 µM FSK in each experiment was used as 100% (average stimulation by 1 µM FSK; 5 ± 2 fold over control). The values represent the means ± S.E. from at least 2 experiments performed in quadruplicate. Expression of sst_2 (C) and sst_5 (D) mRNA levels in a series of transiently co-transfected HEK 293 cells, with a fixed concentration sst_2 cDNA plasmid (10 µg) and increasing concentrations of sst_5 cDNA plasmid (0.1-4 µg). Sst and hprt mRNA levels were quantified by a TaqMan assay and results are depicted as bars, representing the means ± S.E. from 2 separate experiments performed in duplicate, and are adjusted for HPRT expression.

First, in order to validate the CRE-LUC assay, functional properties of the SRIF-analogs to inhibit FSK-stimulated cAMP response in transiently transfected HEK 293 cells, expressing only sst_2 or sst_5, were explored. In

wild type HEK 293 cells, no inhibitory effects of the SRIF-analogs to inhibit FSK-stimulated cAMP response was observed (data not shown). As depicted in figure 1A, in sst_2-expressing HEK 293 cells, a concentration-dependent inhibition of cAMP response was seen for OCT (IC_{50} 0.02±0.08 nM) and SOM230 (IC_{50} 0.6±0.12 nM), while BIM-23206 suppressed FSK-induced cAMP response only at 10-100 nM (IC_{50} 13±0.11 nM). FSK-induced cAMP response in HEK293 cells, expressing sst_5 (Fig.1B) was dose-dependently inhibited by SOM230 (IC_{50} 0.09±0.09 nM) and BIM-23206 (IC_{50} 0.24±0.10 nM), while OCT appeared the least potent (IC_{50} 4.6±0.12 nM). To evaluate whether sst_2 and sst_5 receptor expression can influence the functional properties of each receptor subtype, we compared the potencies of the SRIF-analogs at the physiological 1 nM concentration to inhibit FSK-induced cAMP response in a series of transiently transfected HEK 293 cells, in which a constant sst_2 mRNA expression level was maintained together with increasing sst_5 mRNA expression levels (Fig. 1C and 1D). As shown in figure 2A, OCT suppressed FSK-induced cAMP response to a similar extent independent of the ratio sst_2/sst_5 transfected and, moreover, equalled its potency to suppress cAMP response in sst_2-expressing HEK 293 cells only. On the other hand, the inhibitory effects of BIM-23206 (1 nM) significantly increased with increasing sst_5 expression, i.e. from 9 ± 5% to 38 ± 8% and finally 56 ± 7% ($p < 0.05$; Fig.2B). Moreover, only when sst_5 expression was greater than sst_2 expression, the inhibitory potency of 1nM BIM-23206 was not significantly different as compared with inhibition of FSK-induced cAMP response in the sst_5 mono-transfected HEK 293 cells. Although 1 nM SOM230 already suppressed FSK-induced cAMP response by 51 ± 6% in sst_2-expressing HEK 293 cells, a similar pattern of inhibitory potency as for BIM-23206 was observed for the multiligand SOM230, i.e. inhibition of FSK-induced cAMP response improved when sst_5 expression was increased (Fig. 2C). Subsequently, in order to evaluate whether IC_{50} values of the inhibitory effects of OCT, BIM-23206 and SOM230 on FSK-induced cAMP response would be influenced by different sst_2 and sst_5 mRNA expression levels, full concentration-response experiments were performed. The IC_{50} value for OCT in all sst_2/sst_5 transfectants was comparable to the IC_{50} of

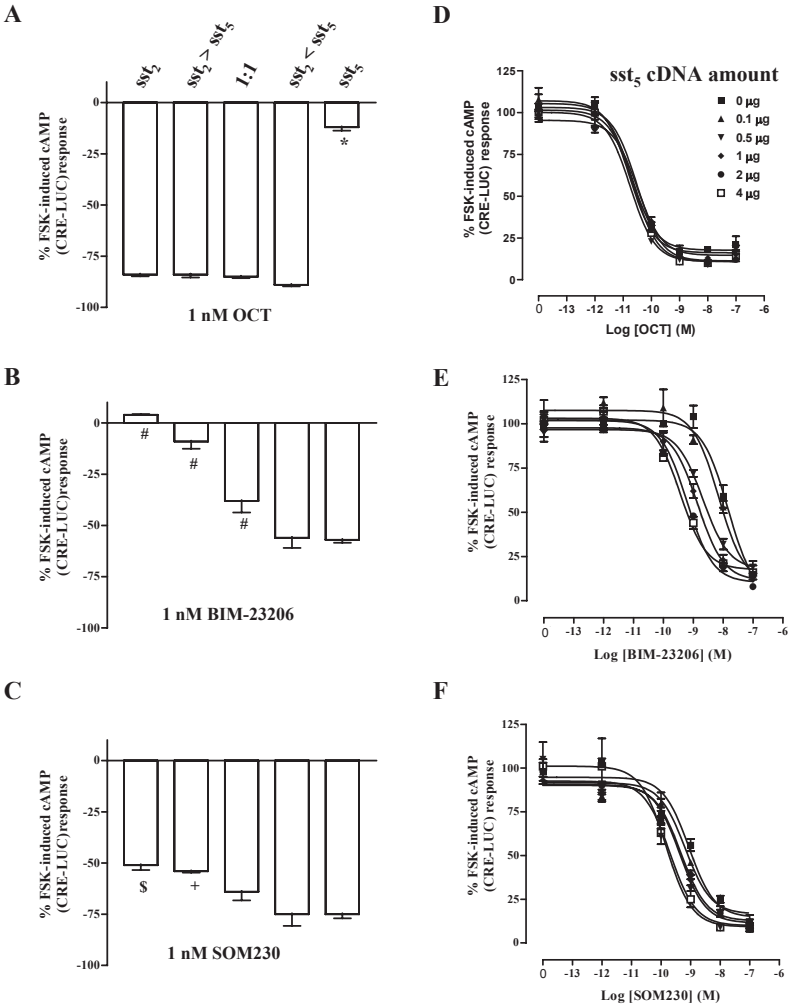

Figure 2. SRIF-agonist (1 nM) induced inhibition of FSK-induced cAMP response in a series of transiently transfected HEK 293 cells, expressing only sst_2 or sst_5 as well as co-expressing both sst subtypes in different ratios. Bars represent the means ± S.E. from 2 separate experiments performed in quadruplicate. A) 1 nM OCT; *, $p<0.01$ sst_5 vs. all other groups: B) 1 nM BIM-23206; #, $p<0.05$ vs. all other groups and C) 1 nM SOM230; $, $p<0.05$ sst_2 vs. 1:1, $sst_2 < sst_5$ and sst_5; +, $p<0.05$ $sst_2 > sst_5$ vs. $sst_2 < sst_5$ and sst_5. Concentration-dependent inhibition of FSK-induced cAMP response by OCT (D), BIM-23206 (E) and SOM230 (F) in a series of transiently co-transfected HEK 293 cells, with a fixed concentration sst_2 cDNA plasmid (10 µg) and enhanced concentrations of sst_5 cDNA plasmid (0.1-4 µg). The data points represent the mean ± S.E. from separate measurements performed in quadruplicate. IC_{50} values are indicated in the results section.

OCT in HEK 293 cells only expressing sst_2 (Fig. 2D). In contrast, increasing sst_5 expression, while sst_2 expression remained at a similar level,

induced the concentration-response curve of BIM-23206 to shift to the left, indicating increased potency. The IC_{50} shifted 40-fold from 15 ± 0.14 nM without sst_5 to 0.37 ± 0.09 nM in the presence of the highest expression of sst_5 ($p<0.05$; Fig. 2E). Apparently, only when sst_5 level exceeds sst_2 expression, the concentration-response curve was comparable to the dose-dependent inhibitory effects of BIM-23206 on FSK-induced cAMP response in HEK 293 cells only expressing sst_5. The concentration-response curve of SOM230 shifted 8-fold to the left as well, although the difference was not statistically significant (Fig. 2F). Its corresponding IC_{50} value of 0.10 ± 0.05 nM at the lowest sst_2/sst_5 ratio, i.e. the highest sst_5 expression, was the only IC_{50} value that reached a comparable value as for the sst_5 mono-transfectant for which SOM230 has a superior membrane binding affinity. On the other hand, in all sst_2/sst_5 ratio levels tested, IC_{50} values of SOM230 were below 1 nM. In addition, a significant negative correlation was observed for the IC_{50} of BIM-23206 in relation to the amount of sst_5 mRNA expressed in this series of co-transfected HEK 293 cells ($r=-0.89$, $p=0.02$). The correlation coefficient for SOM230 was -0.69 ($p=0.13$), while no correlation was observed when the IC_{50} value of OCT was correlated with sst_5 mRNA expression ($r=0.42$, $p=0.41$)

Radioligand binding studies

First, saturation binding experiments in a series of transiently transfected HEK 293 cells with different sst_2 and sst_5 cDNA concentrations were performed and compared with the corresponding mRNA data (Fig. 3). In wild type HEK 293 cells, saturation binding revealed no expression of sst receptors (data not shown). When HEK293 cells expressed more sst_2 than sst_5 mRNA or when these cells co-expressed an approximately 1:1 mRNA ratio, as can be seen in figure 3E and 3F (B_{max} 428±127 fmol/mg protein, Kd 0.19±0.1 nM and B_{max} 277±19 fmol/mg protein, Kd 0.16±0.03 nM, respectively), scatchard analysis showed one set of sst membrane binding sites. As depicted in figure 3G, two sets of sst membrane binding sites, however, were visualized if HEK 293 cells were transfected with superior concentration of sst_5 cDNA compared with

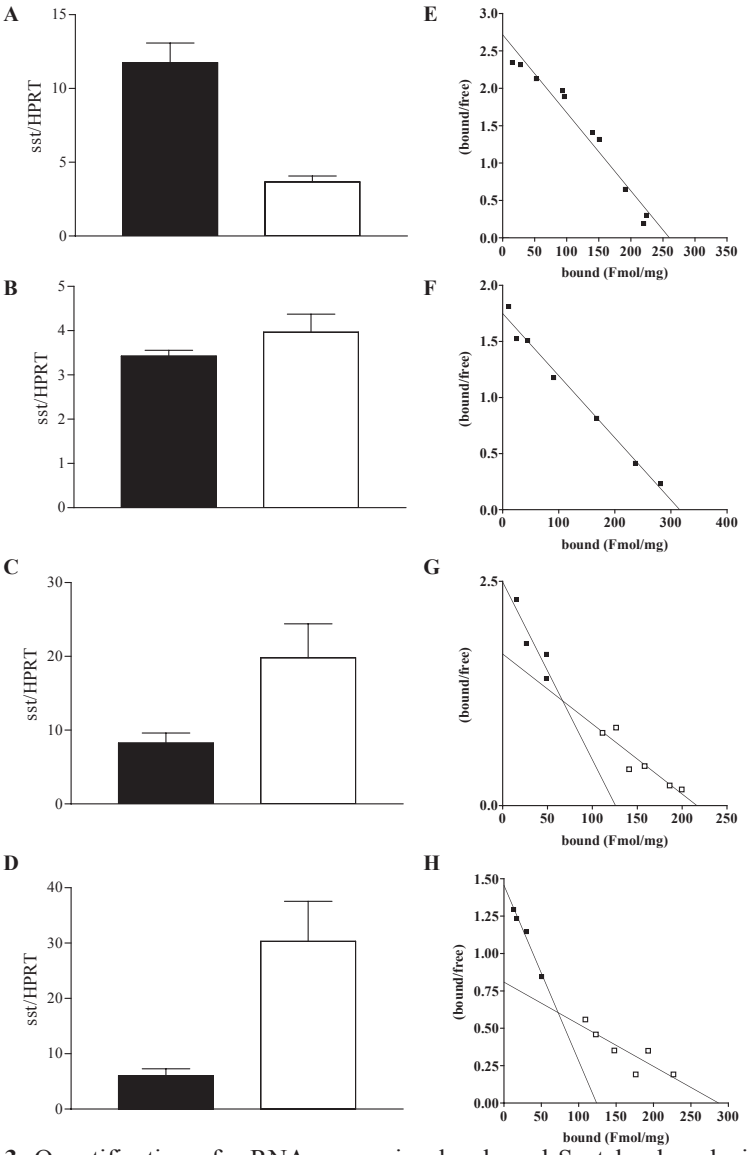

Figure 3. Quantification of mRNA expression levels and Scatchard analysis of [^{125}I-Tyr^{11}]-SRIF-14 binding in a series of transiently co-transfected HEK 293 cells, with different expression ratios of sst_2 and sst_5. A-D) Sst_2 (black bars) and sst_5 (open bars) mRNA levels, together with hprt mRNA levels, were quantified by a TaqMan assay and results are depicted as bars, representing the means ± S.E. from 2-3 separate experiments performed in duplicate, and are adjusted for hprt expression. E-H) Scatchard analysis using [^{125}I-Tyr^{11}]-SRIF-14 was performed to determine the binding affinity (Kd) and total receptor number (Bmax). Each figure is a representative of 2-3 individual experiments. The calculated (mean ± S.E.) Kd and Bmax of each co-transfectant, are described in detail in the Results section. ■, "sst_2-like" binding sites; □, "sst_5-like" binding sites.

sst_2 cDNA ("sst_2-like", B_{max} 98±16 fmol/mg protein and Kd 0.09±0.02 nM; "sst_5-like", B_{max} 202±30 fmol/mg protein and Kd 0.3±0.09 nM), which appeared even more pronounced when sst_5 cDNA concentration was enhanced (Fig. 3D and 3H: "sst_2-like", B_{max} 124±25 fmol/mg protein and Kd 0.10±0.08 nM; "sst_5-like", B_{max} 287±35 fmol/mg protein and Kd 0.35±0.10 nM). The difference in Kd for the "sst_2-like" binding sites compared with the Kd for "sst_5-like" membrane binding sites seem well in agreement with the reported (slightly) better membrane binding affinity of SRIF-14 for sst_2 compared with sst_5 [Table I; (17, 25-28)].

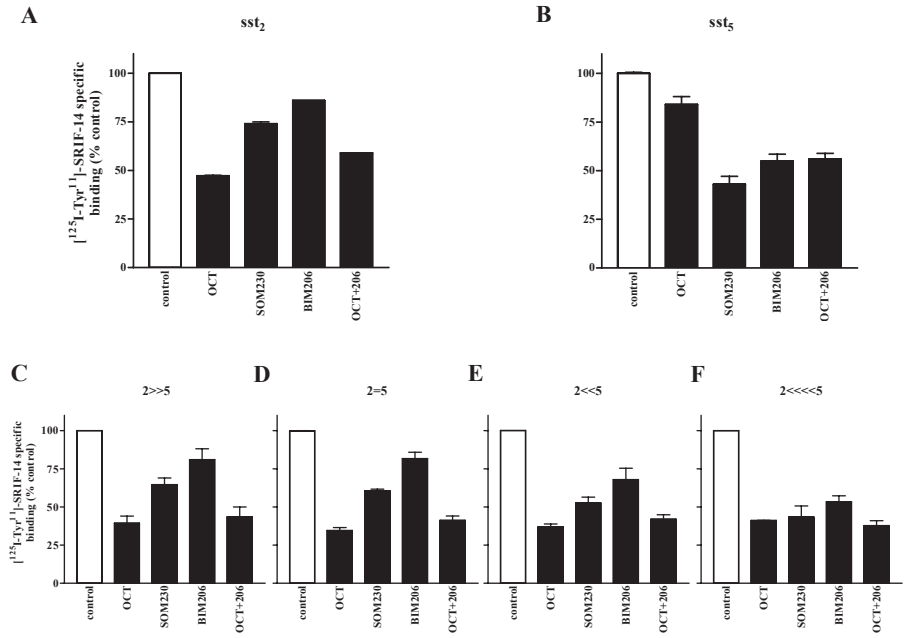

Figure 4. Competition experiments for [^{125}I-Tyr^{11}]-SRIF-14 specific binding sites on isolated cell membranes from sst_2 (A), sst_5 (B) and from four different sst_2/sst_5 ratio-expressing (C-F) HEK 293 cells. 50 µl of membrane preparations of transiently transfected HEK 293 cells were incubated in a total volume of 100 µl at room temperature for 45 min with 25 µl [^{125}I-Tyr^{11}]-SRIF-14 (40.000cpm) and either 25 µl binding assay buffer (total binding = 100%) or with 1 nM OCT, SOM230, BIM-23206 or OCT and BIM-23206. After the incubation, 1 mL ice-cold Hepes buffer was added to the reaction mixture, and membrane-bound radioactivity was separated from unbound by centrifugation during 2 min at 14,000 rpm in a Eppendorf microcentrifuge. The remaining pellet was washed twice in ice-cold Hepes buffer, and the final pellet was counted in a γ-counter. Bars represent the means ± S.E. from 2-3 separate experiments performed in triplicate for each transfection with sst_2 or sst_5. When error bars are not apparent the S.E. values were smaller than symbol size.

Competition experiments for [^{125}I-Tyr^{11}]-SRIF-14 binding sites on isolated cell membranes from HEK293 cells expressing four different sst_2/sst_5 ratios (Fig. 3A-D) by the SRIF-analogs at the physiological concentration of 1 nM were compared with their potencies in the sst_2 and sst_5 mono-transfectants. Figure 4A, in agreement with the receptor binding affinities of the SRIF-analogs for sst_2, demonstrates that the order of potency to displace [^{125}I-Tyr^{11}]-SRIF-14 binding sites in sst_2-expressing HEK 293 cells was: OCT =/> OCT+BIM-23206 > SOM230 > BIM-23206. When the same analysis was carried out for the sst_5-expressing cells (Fig. 4B), the order of potency to displace [^{125}I-Tyr^{11}]-SRIF-14 binding sites appeared: SOM230 > BIM-23206 = OCT+BIM-23206 > OCT, which was in accordance with the sst_5-binding affinities of the SRIF-analogs. As for the co-transfectants, OCT at 1 nM remained the most potent SRIF-analog throughout all four situations to displace [^{125}I-Tyr^{11}]-SRIF-14 binding sites (Fig. 4C-F) and showed the same displacement efficacy as of OCT in the sst_2 mono-transfectant. On the other hand, 1 nM BIM-23206 demonstrated displacement of [^{125}I-Tyr^{11}]-SRIF-14 binding similar to the sst_5 mono-transfectant (-45 ± 3%) only when sst_5 expression was superior compared with sst_2 expression (-19 ± 2%, fig. 4C; -19 ± 2%, fig. 4D; -32 ± 2%, fig. 4E and -47 ± 2%, fig. 4F; $p<0.05$). As already demonstrated for the cAMP response experiments, SOM230 showed a comparable pattern as BIM-23206 in the radioligand competition experiments although to a considerably lesser extent. Moreover, 1 nM SOM230 was most potent to displace [^{125}I-Tyr^{11}]-SRIF-14 binding sites when sst_5 had the highest expression level (Fig. 4F, -57 ± 2%) and once more, only in this latter co-transfectant the efficacy of SOM230 equalled its efficacy in the sst_5 mono-transfectant (-57 ± 3%) as well as the efficacy of OCT (-59 ± 1%). Co-treatment with OCT and BIM-23206 at a final concentration of 1 nM was as efficacious as 1 nM OCT in all co-transfectants.

The sst_2 antagonist BIM-23454 in sst_2+sst_5 expressing HEK 293 cells

We also evaluated the functional responses of the SRIF-analogs in the

sst_2/sst_5 co-transfected HEK293 cells, when sst_2 expression was functionally blocked. Therefore, we used the sst_2 antagonist BIM-23454 in the CRE-LUC assay. We used a 50 nM concentration for the sst_2-antagonist, which is sufficient to bind sst_2 but not sst_5, because the binding affinity is 32 nM and 138 nM, respectively. As depicted in figure 5, 50 nM BIM-23454 did not alter FSK-induced CRE-LUC gene activation, both in sst_2 (Fig. 5A) and in sst_5 (Fig. 5B) expressing HEK 293 cells. In the presence of BIM-23454, the dose-dependent inhibition of FSK-induced cAMP response by OCT in sst_2-expressing HEK 293 cells, shifted 40-fold to the right, indicating decreased potency (Fig. 5C). How-

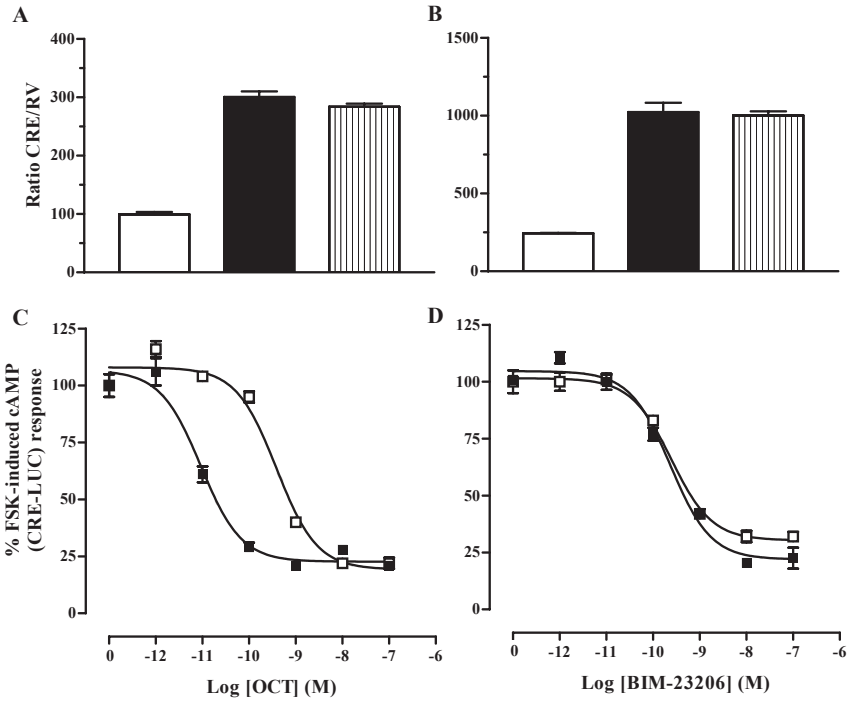

Figure 5. Control experiments for BIM-23454 in HEK-293 cells, only expressing sst_2 or sst_5. Effect of 50 nM BIM-23454 on FSK (1µM)-induced activation of CRE-LUC reporter gene construct, corrected for RSVlacZ (21) expression in sst_2 (A) or sst_5 (B) expressing HEK 293 cells. Control, open bars; FSK, black bars and FSK+ 50 nM BIM-23454, hatched bars. Bars represent mean ± SE from two individual experiments, performed in quadruplicate. Concentration-effect curves of OCT and BIM-23206 on FSK-induced cAMP response in sst_2 (C) or sst_5 (D) expressing HEK 293 cells, respectively, in the presence (□) or absence (■) of 50 nM BIM-23454. Each graph is representative for two individual experiments, run in quadruplicate.

ever, the dose-dependent inhibition of FSK-induced cAMP response by BIM-23206 in sst_5-expressing HEK 293 cells was not affected (Fig. 5D) Subsequently, in a series of transiently transfected HEK 293 cells, in which mRNA expression levels were comparable for sst_2 and sst_5, the dose-dependent inhibition of FSK-induced cAMP response by OCT shifted 45-fold to the right, indicating decreased potency (Fig. 6A; IC_{50} 0.04 ± 0.09 nM in the absence and 1.8 ± 0.1 nM in the presence of 50 nM BIM-23454). The inhibition of FSK-induced cAMP response by 1 nM OCT was

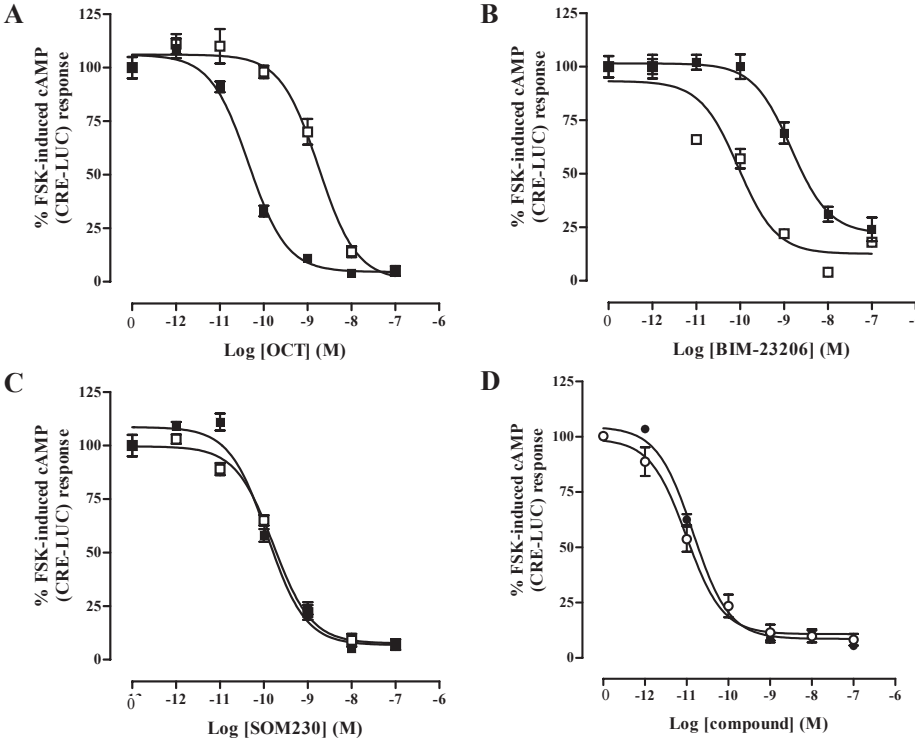

Figure 6. Effect of BIM-23454 on concentration-effect curves of OCT (A), BIM-23206 (B) and SOM230 (C) on FSK-induced cAMP response in HEK 293 cells, expressing equal amounts of sst_2 (10.1 ± 1.3 copies sst_2/HPRT) and sst_5 (10.3 ± 1.1 copies ss_{t5}/HPRT) mRNA. Each graph is representative for two individual experiments, run in quadruplicate. (■) Control curve and (□) + 50 nM BIM-23454. (D) Dose-dependent inhibition of FSK-induced cAMP response by Cabergoline in transiently transfected HEK 293 cells, expressing D2R (●) or D2R and sst_2+sst_5 (○; 9.2 ± 4.4 copies D2R/HPRT, 6.5 ± 5.1 copies sst_2/HPRT and 3.5 ± 2.3 copies sst_5/HPRT). The values represent the means ± S.E. from at least 2 separate experiments run in quadruplicate.

significantly lowered in the presence of BIM-23454 (89 ± 1% vs 30 ± 3% suppression, $p<0.05$). However, the dose-dependent inhibition of FSK-induced cAMP response by BIM-23206 shifted 15-fold to left when HEK 293 cells were co-treated with BIM-23454 (Fig. 6B; IC_{50} from 1.5 ± 0.05 nM to 0.1 ± 0.3 nM), indicating increased potency. Also, the inhibition of FSK-induced cAMP response by 1 nM BIM-23206 was significantly enhanced in the presence of BIM-23454 (31 ± 5% vs 78 ± 2% suppression, $p<0.05$). The corresponding IC_{50} for the dose-dependent inhibition of FSK-induced cAMP response in sst_5+sst_5 expressing HEK 293 cells by SOM230 remained 0.1 nM in the presence of BIM-23454, while the inhibition at 1 nM SOM230 of FSK-induced cAMP response was almost 80% in the presence as well as in the absence of BIM-23454 (Fig. 6C).

Cabergoline, a dopamine D2 receptor (D2R) agonist in sst_2+sst_5+D2R expressing HEK 293 cells

To evaluate whether the bio-availability of G-proteins could be a limitation in our experimental design, we set up a final series of experiments in which we introduced the Dopamine D2 receptor into co-transfected the D2R with sst_2 and sst_5. As shown in figure 6D, the concentration dependent inhibition of FSK-induced cAMP response by Cabergoline, a D2R-agonist, was not affected when D2R was co-expressed with both sst_2 and sst_5 (IC_{50} of CAB in the presence and absence of sst_2+sst_5 was 0.01 ± 0.09 nM vs 0.01 ± 0.06 nM, respectively).

Discussion

In the present study, the pharmacological profiles of several SRIF-analogs as determined by inhibition of FSK-induced cAMP response and radioligand binding in HEK 293 cells expressing different sst_2/sst_5 ratios, provide quantitative evidence to support the concept that the sst_2 is a functional dominant receptor subtype. While several reports have evaluated the inhibitory efficacy of sst_2 and sst_5 activation in the regulation of GH secretion by use of primary cultures of GH-secreting pituitary adenomas (15, 28-30), this report is the first that investigated in a basic experimental design,

whether these two sst subtypes have a functional interplay with each other. The CRE-LUC assay, a reporter gene to quantify cAMP release, has been used frequently to analyze functional responses of different GPCR's in transfected cell systems (20, 31, 32). Our observed functional responses (IC_{50}) of the SRIF-analogs in the HEK 293 cells, only expressing sst_2 or sst_5, are well in agreement with the sst membrane binding affinities of the different SRIF-analogs reported in literature (9, 27), *i.e.* OCT preferentially acts via sst_2, SOM230 is bi-selective for sst_2 and sst_5 but has superior (binding and functional) affinity properties for sst_5, and BIM-23206 is 100-fold more sst_5-specific as compared with sst_2 (Table 1). Subsequently, by evaluating the functional responses and the displacement of [^{125}I-Tyr^{11}]-SRIF-14 binding sites by OCT, BIM-23206 and SOM230 in a series of sst_2/sst_5 co-transfectants , it is suggested that sst_5 activation by sst_5-specific SRIF analogs can only occur if sst_5 subtype expression significantly exceeds that of sst_2 expression. Apparently, a substantial part of available BIM-23206 is bound to sst_2, which explains its attenuated efficacy to suppress FSK-induced cAMP response. SOM230, with a nanomolar binding affinity for sst_2 but with a superior sub-nanomolar binding affinity for sst_5 is already efficacious when sst_2 expression is higher than sst_5 expression but induces optimal functional responses when sst_2/sst_5 expression ratios are low. Strikingly, a dominant sst_2-mediated suppression, illustrated by OCT, is suggested as well as by the superior activity of OCT at low sst_2/sst_5 expression ratios with respect to inhibition of FSK-induced cAMP release, as well as displacement of [^{125}I-Tyr^{11}]-SRIF-14 binding sites. OCT-mediated effects are not sensitive for the degree of sst_5 expression, as demonstrated by the absence of a correlation between the IC_{50} values of OCT and the expression levels for sst_5, while the completely opposite is seen for BIM-23206. Therefore, our data support the observation that individual sst receptors in transfected cell systems have a good correlation between membrane binding affinity and functionality (33), but when two sst subtypes are co-expressed, a functional interplay, i.e. dominance by sst_2 over sst_5, is observed. Finally, scatchard analysis of the co-transfectants, forms a third part of evidence in support of our hypothesis. The density of endogenous sst expression in receptor-rich tis-

sues such as brain, pituitary and pancreas measured with a non-selective sst radioligand ^{125}I-LTT-SRIF-28 (which detects all five sst subtypes) ranges between 220 and 360 fmol/mg in rats (34, 35). Because receptor over-expression might form an artefact in interpreting functional relationship between sst receptors (36), the total sst subtype expression obtained in our co-transfectants, 277-428 fmol/mg protein with a transfection efficiency of approximately 35%, is within the range (107-2500 fm/protein) of sst receptor densities used in numerous reports that investigated SRIF receptor function (37-42). While scatchard analysis to calculate Kd and Bmax of the high sst_2/sst_5 and 1:1 ratio co-transfectants resulted in a single class of sst-binding sites, two distinct sets of binding sites became apparent only when sst_5 expression was superior to sst_2 expression. In the latter co-transfectants, the lower Bmax with the lower and thus better Kd, may represent the "sst_2-like" subtype, while enhanced "sst_5-like"expression is supported by higher Bmax values and a slightly higher Kd, which is well in agreement with the mRNA status of the co-transfectants. So, two sets of sst-binding sites are found only when sst_5 mRNA expression is expressed at superior level compared with sst_2 mRNA. By the use of sst subtype selective SRIF-analogs, it has been demonstrated that both sst_2 and sst_5 selective agonists suppress GH secretion (28, 29) in primary cell cultures from normal anterior pituitaries, as well as from GH-secreting pituitary adenomas. In agreement with the latter, sst_2 and sst_5 receptors are the predominantly expressed SRIF receptor subtypes, both at the mRNA (7, 13, 14) and the protein level (6) in primary cultures of GH-secreting pituitary adenomas. The current clinically used sst_2-preferring SRIF analogs, OCT and lanreotide, have been successful in the treatment of acromegaly because of the high percentage of tumors expressing a significant amount of sst_2. In addition, while several groups demonstrated that the sst_5 subtype seems to be expressed at a higher level as compared with sst_2, only sst_2 mRNA expression in GH-secreting pituitary adenomas shows a positive correlation with the *in vivo* GH suppression induced by an acute test using a single injection of 200µg OCT (13), as well as with the *in vitro* and *in vivo* responsiveness to OCT in another series of patients (14). Surprisingly, although

SOM230 shows a very high affinity for sst_5 receptors, no significant correlation was found between the *in vitro* GH suppression by a maximally active concentration of SOM230 and sst_5 mRNA levels, whereas a positive correlation was found between the effects of SOM230 and sst_2 mRNA levels (14). Although these data were unexpected, a previous study by Jaquet and coworkers in fact already made the same observation using SRIF-14 (13). Moreover, Cervia and coworkers showed in AtT-20 cells, endogenously expressing sst_2 and sst_5 subtypes that most SRIF-analogs will act via sst_2 receptors, even if they are capable of acting via sst_5 receptors (43). These observations are well in agreement with our data in transiently sst_2+sst_5 transfected HEK 293 cells and support the hypothesis that the sst_2 receptor is functionally dominant over sst_5. Functional association of sst_2 and sst_5 has been suggested to mediate additive GH inhibition, at least in human fetal pituitary cell cultures (30). In this study Ren and coworkers used a sst_2 selective antagonist that was capable of reversing the GH suppressive effects of 10 nM of sst_2/sst_5 biselective agonists, or that of sst_2 and sst_5 agonists (10 nM) in combination. Our results with SOM230, as well as with the co-treatment of OCT and BIM-23206 at the physiological concentration of 1 nM do not show an enhanced functionality to inhibit adenylyl cyclase, nor to displace sst membrane binding sites. If SOM230 would induce functional association of both subtypes, maximal effects would have occurred in other co-transfectants than the lowest sst_2/sst_5 ratio co-transfectant as well. Previous studies already demonstrated that SOM230 inhibits GH release in a higher number of GH-secreting pituitary adenomas compared with OCT, both *in vitro* (14, 44) and *in vivo* (18), but not with a better efficacy in terms of maximal GH-suppressive effect or lower IC_{50} value. Nevertheless, although sst_2 seems the dominant receptor subtype as new SRIF-analogs with enhanced sst_2 binding affinities inhibit GH secretion more potently compared to OCT, SOM230 is able to lower GH levels in both OCT-responders (via sst_2) and partial responders (via sst_5) and, therefore, SRIF analogs bispecific for sst_2 and sst_5 have a clear advantage over OCT and might increase the number of acromegalic patients that can be biochemically controlled during long-term medical treat-

ment. Our experiments with the sst_2 antagonist BIM-23454, demonstrate indeed that SOM230 can still be effective when sst_2 availability is low (as in those acromegalic patients in which OCT is ineffective). In the presence of BIM-23454, both OCT and BIM-23206 drift towards their sst_5 binding affinity and their IC_{50} values are comparable with the IC_{50} values observed in the sst_5-transfectants. These data confirm that OCT is not effective when sst_2 is expressed at a low level, i.e. cannot lower GH in the subgroup of acromegalic patients that are (partially) non-responders for OCT, but SOM230 can still be efficacious to lower FSK-induced cAMP response because it now can function via sst_5 receptors. A critical reflection regarding our observations, reveals that up till now, no intracellular regulatory mechanism is investigated that can account for the functional dominance of sst_2. Studies which describe GPCR signal transduction, especially with respect to individual sst subtype binding to G-proteins, seem crucial to understand the physiological rationale behind the sst_2 dominance hypothesis. However, the results with Cabergoline (CAB), a dopamine agonist which has superior binding affinity for the G-protein coupled D2 receptor (45), show that the efficacy of CAB is not affected when sst_2+sst_5 receptors are being co-expressed together with the G-protein coupled D2R. These data strongly suggest that the amount of G-protein available in our experimental design is not a limiting factor causing the observed functional interplay between sst_2 and sst_5. Nevertheless, a further understanding of the stoichiometry of ligand-receptor and G-protein complex by GTPγS binding-, co-immunoprecipitation- and/or fluorescence tagged receptor- studies can be of experimental help in this matter (46-48). In conclusion, our report is the first that investigated the functional interplay between the key-players in the regulation of human GH release, *i.e.* the sst_2 and sst_5 subtype. The sst_2 appears to function in a dominant way over sst_5, while sst_5 itself can only serve at maximal potency when sst_2/sst_5 expression ratio is low. The multiligand SOM230 can mediate GH inhibition via both sst_2 and sst_5, which make SRIF-analogs targeting both sst subtypes suitable candidates to increase the number of acromegalic patients biochemically controlled.

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Chapter IV-2

RNA interference as a novel tool to study somatostatin receptor function

Abstract

Short interfering RNAs (siRNAs) resulting into silencing of specific genes by double-stranded RNA (dsRNA), have been developed for systematically deciphering the functions of protein coding genes of the human genome. We have used the mammalian expression vector pSUPER, that directs the synthesis of siRNA-like transcripts, to suppress somatostatin receptor subtype (sst) 2 and sst_5 gene expression in mammalian cell lines. Two designed siRNAs for the sst_2 gene potently lowered mRNA expression levels in stably sst_2-expressing CHO-K1 cells (74-75% suppression, $p<0.05$ vs control and scrambled). The siRNA-sst_5 plasmid, transiently transfected in sst_5-expressing HEK 293 cells, potently suppressed sst_5 mRNA expression levels (>75%). In conclusion, we have demonstrated that the synthesized siRNA constructs for the sst_2 and sst_5 gene have the merit to become an interesting novel tool to evaluate the individual role of endogenously expressed sst receptors in primary cells.

Introduction

Studies on protein function through the inhibition of their action have been critical for our understanding of many normal and abnormal biological processes. Numerous approaches, ranging from small molecule antagonists to knockout animals, have resulted into substantial advances in our understanding of the function of many proteins. Still, a lack of specificity or restricted applicability has limited their utility. The discovery that long double-stranded RNA molecules (dsRNA) can induce sequence-specific silencing of gene expression in primitive organisms, such as *Caenorhabditis elegans* and *Drosophila melanogaster*, revealed a previously unknown mechanism of gene regulation which is highly conserved throughout multicellular organisms (1, 2). This process is called RNA interference (RNAi) which mediates gene silencing via two main steps: (i) the dsRNA is initially recognised by an enzyme of the RNAse III family of nucleases, named Dicer (the enzyme that normally produces siRNA in vivo), and processed into small double-stranded molecules, termed siRNA and (ii) the siRNAs are bound by the RNA-induced silencing complex (RISC), which is a multi-protein complex (with RNase activity) that guides the targeted RNA to degradation (3).

However, in most mammalian cells the introduction of dsRNA provokes a strong cytotoxic response (4, 5). By synthesizing short interfering RNAs (siRNA) this non-specific effect can be circumvented and a transient reduction in gene expression can be observed. To overcome the limitation in the duration of the effect, a new mammalian expression vector that directs the synthesis of siRNA-like transcripts [pSUPER] was designed (6). It was demonstrated that siRNA expression mediated by this vector caused efficient and specific down-regulation of gene expression, resulting in functional inactivation of the targeted genes (6). Somatostatin (SRIF), a cyclic peptide produced in the hypothalamus, throughout the central nervous system, as well as in most major peripheral organs, inhibits hormone release from the anterior pituitary gland, pancreas and the gastro-intestinal tract (7, 8). Five different SRIF receptor subtypes (sst_{1-5}) have been identified (9, 10). These receptor subtypes are variably expressed in the SRIF-target tissues (11, 12). Sst bind with varying affinity to the different SRIF-analogs, which were synthesized for clinical application (13, 14) as well as for basic science (15, 16), thereby providing a tool to unravel the (patho-) physiological function of the five sst's. We have used the pSUPER vector to suppress sst_5, transiently transfected in mammalian cells, in order to evaluate if RNAi can be of use to study the individual role of sst_2 and sst_5 in the regulation of growth hormone (GH) release from primary cultures of GH-secreting pituitary adenoma cells.

Methods

Cell culture

For expression of sst_2 and sst_5 in Chinese hamster ovaries (CHO)-K1 cells, human sst_2 and sst_5 cDNA in pBluescript (pBS) (a kind gift of G.I. Bell, Howard Hughes Medical Institute Chicago, Illinois) was excised from pBS and inserted into the Nhe-1/Sal1 or EcoRI/XbaI cloning site, respectively, of the retroviral expression vector pCi-neo. Selection was made by the geneticine resistance gene (G418). This vector was used to stably transfect (using DOTAP) CHO-K1 cells. Stably transfected CHO-K1 cells were selected and cultured in nutrient mixture F12 (HAM) me-

dium [supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), fungizone (0.25 µg/mL) and 10% FCS + geneticine (0.5 mg/mL)]. HEK 293 cells (kind gift of Dr. A.P.N. Themmen, Internal Medicine, Erasmus MC Rotterdam, the Netherlands) were cultured in DMEM/F-12 medium, supplemented with 10% fetal calf serum (FCS), penicillin ($1*10^5$ U/L), streptomycin (50 mg/L), fungizone (0.25 mg/L) and L-glutamine (2 mmol/L). The cells were maintained in 75 cm^2 flasks at 37°C in a CO_2-incubator and routinely passaged by trypsinization as described in detail previously (17). Media and supplements were obtained from GIBCO Bio-cult Europe (Invitrogen, Breda, the Netherlands).

Preparation of siRNA

Three different siRNAs for sst_2, one siRNA for sst_5 and one scrambled control were designed. The predicted structures of the synthetic siRNA constructs are depicted in Figure 1. The 19nt target sequence for sst_5 (H-219) and sst_2 (H-121) were flanked in the mRNA with AA at the 5' end of the sense strand. Regions at the mRNA to select all the 19nt from were within the coding region: 100 bp from start and termination of translation. GC richness of all 19nt targeting sequence was more than 30%. To prevent premature termination of the transcript, the 19nt that were selected must not contain a stretch of four or more Adenines or Thymidines. Furthermore, two siRNA constructs for sst_2, H-523 and H-573, were selected for a high Adenine and/or Thymidine content at the 5' end of the antisense strand (18). Coding sequences for siRNA were analyzed by BLAST research at the National Center for Biotechnology Information against all human sequences deposited in the GenBank and RefSeq data-bases and were not found to have significant homology to genes other than the targets. Oligonucleotides were synthesized by Sigma (Zwijndrecht, The Netherlands). Double-stranded siRNA was prepared as described previously (6). Briefly, single stranded oligonucleotides were annealed with corresponding complementary single-stranded DNA oligonucleotides, separated by a short hairpin spacer. The resulting transcript is predicted to fold back on itself to form a 19-base pair stem loop

(Fig.1). The resultant dsDNA hairpin was inserted into Bgl II-HindIII site of pSUPER-H1 to generate siRNA plasmids. DH5α competent cells were transformed with the resultant plasmids. Plasmid DNA was purified using a commercial DNA purification kit (QIAGEN).

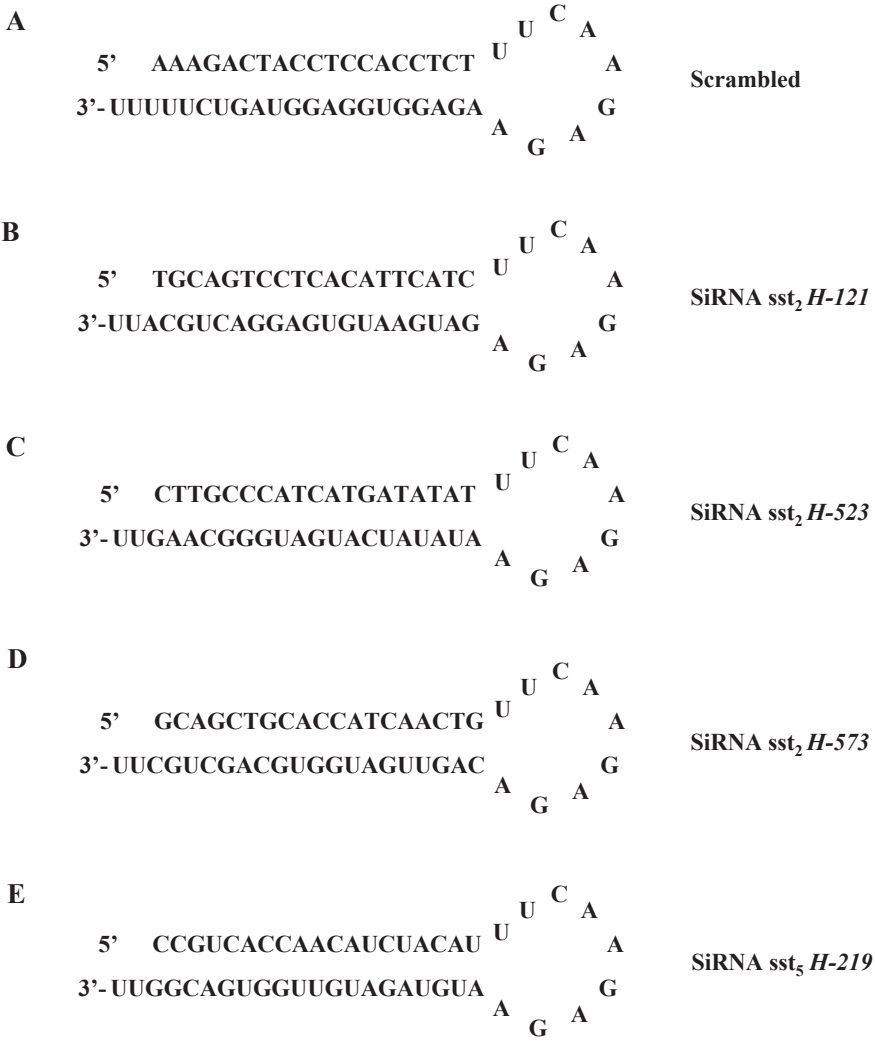

Figure 1. Structure of synthetic siRNA constructs. The numbers in italic indicate the nucleotide position at which the 5' end of the sense strand of the siRNA matches the sst gene reference sequence. The scrambled construct was created on a random basis. In general, the 19nt sequences from the target transcript are separated by a short spacer from the reverse complement of the same sequence, predicted to fold back on itself to form a 19 base pair stem loop structure, i.e. short hairpin siRNA (6).

Transfection of sst_2-expressing CHO-K1 cells and mRNA analysis

Batches of $1x10^6$ stably sst_2-expressing CHO-K1 cells were used in each transfection experiment with the Nucleofector™ (AMAXA Biosystems, Germany). Harvested cells were spin down at 1800 rpm for 5 min, and the medium removed. Cells were then resuspended in 100µl Nucleofector™ solution T (AMAXA Biosystems) at room temperature followed by addition of 2 µg RNAi H-121, RNAi H-523, RNAi H-573, scrambled or pSUPER cDNA. The mixture of cells, solution and cDNA was transferred to a 2 mm electroporation cuvette (AMAXA Biosystems) inserted in the Nucleofector™ and program U-23 (>90% transfection efficiency) used for transfecting the cells. Immediately after transfection, 500µl of medium was added to the cells to reduce damage of the cells. Finally, $1x10^5$ transfected cells were resuspended in a total volume of 1mL in 24-well plates. After 72 hr of incubation, cells were lysed and collected for quantitative RT-PCR of sst_2, as described below.

Transfection and quantitave RT-PCR in HEK 293 cells

Before transfection, wild-type HEK 293 cells were seeded at 40% confluence in 75 cm^2 flasks and transfected the next day using the calcium phosphate precipitation method. HEK 293 cells were transiently transfected with various concentrations of human sst_5 cDNA as well as 5µg RNAi cDNA or carrier cDNA. Quantitative PCR was performed as described previously (19). Messenger RNA was isolated using Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway) from transiently transfected (72hr) wild-type HEK 293 cells. The cells were lysed for 2 min in an ice-cold Tris-buffer (100 mM Tris-HCl, pH 8, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM DTT and 5 U/100µl RNAse inhibitor (HT Biotechnology Ltd., Cambridge, UK). The mixture was centrifuged at 14,000 rpm for 1 min to remove cell debris. After adding 40 µl pre-washed Dynabeads Oligo $(dT)_{25}$ to the supernatant, the mixture was incubated for 5 min on ice. Thereafter, the beads were collected with a magnet, washed three times with a Tris-buffer (10 mM Tris HCl, pH 8, 0.15 M LiCl, 1 mM

EDTA, 0.1% LiDS), and once with a similar buffer from which LiDS was omitted. Messenger RNA was eluted from the beads in 2 x 20 µl H_2O for 2 min at 65 °C. Complementary DNA (cDNA) was synthesized using the poly A^+ mRNA in a Tris-buffer (50 mMTris-HCl, pH 8.3, 100 mM KCl, 4 mM DTT, 10 mM $MgCl_2$) together with 1 mM of each deoxynucleotide triphosphate, 10 U RNAse inhibitor, and 2 U AMV Super Reverse Transcriptase (HT Biotechnology Ltd., Cambridge, UK) in a final volume of 40 µl. This mixture was incubated for 1 h at 42 °C. One tenth of the cDNA library was used for quantification of sst subtype mRNA levels. The assay was performed using 15 µl TaqMan Universal PCR master mix (Applied Biosystems, Capelle aan de IIssel, The Netherlands), 500 nM forward primer, 500 nM reverse primer, 100 nM probe and 10 µl cDNA template, in a total reaction volume of 25 µl. The reactions were carried out in a ABI 7700 sequence detector (The Perkin-Elmer Corporation, Foster City, CA). PCR amplification started with a first step for 2 min at 50 °C, followed by an initial heating at 95 °C for 10 min and, subsequently, samples were subjected to 40 cycles of denaturation at 95 °C for 15 sec and annealing for 1 min at 60 °C. To ascertain that no detectable genomic DNA was present in the poly A^+ mRNA preparation, since sst genes are intron-less, the cDNA reactions were also performed without reverse transcriptase. The detection of human hypoxanthine-phosphoribosyl-transferase (hprt) mRNA in HEK293 cells served as a control and was used for normalization of the sst subtype mRNA levels. The primer sequences that were used have been described in detail previously (20, 21).

Statistical analysis

The statistical significance was determined by using one-way analysis of variance (ANOVA). When significant overall effects were obtained by the ANOVA method, comparisons were made using Newman-Keuls multiple comparisons test. Data are reported as means ± SEM of the indicated n values.

Results

First, three siRNA-sst_2 plasmids were tested, compared with scrambled, in

stably expressing sst_2-CHO-K1 cells that were transfected via electroporation by the Nucleofector™ technology. As can be seen in figure 2, silencing at the mRNA level occurred with varying efficacy (24-75% suppression of sst_2/HPRT mRNA expression), while both the empty pSuper-H1 vector and the scrambled construct did not alter sst_2/HPRT mRNA expression.

siRNA efficacy was for sst_5 explored on sst_5 mRNA expression with various concentrations of sst_5 cDNA. When 5µg sst_5 cDNA was used, the siRNA-sst_5 plasmid suppressed sst_5 mRNA expression by 75% in transiently transfected sst_5-HEK293 cells (Fig. 3A). moreover, when the concentration of sst_5 cDNA, co-transfected in HEK 293 cells, was lowered 10-fold, siRNA at the mRNA level showed a 92% reduction of sst_5 expression (Fig. 3B).

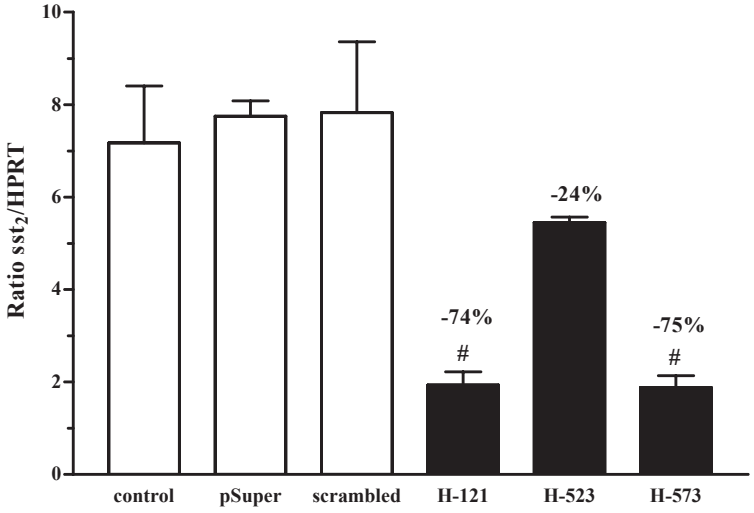

Figure 2. Expression of sst_2 mRNA levels in a series of sst_2-expressing CHO-K1 cells transiently transfected (72hr, by NucleofectorTM) with three different siRNA constructs for sst_2 (2µg). Sst and HPRT mRNA levels were quantified by a TaqMan assay and results are depicted as bars, representing the means ± S.E. from two experiments performed in quadruplicate, and are adjusted for HPRT expression. #, $p<0.05$ compared with control and scrambled.

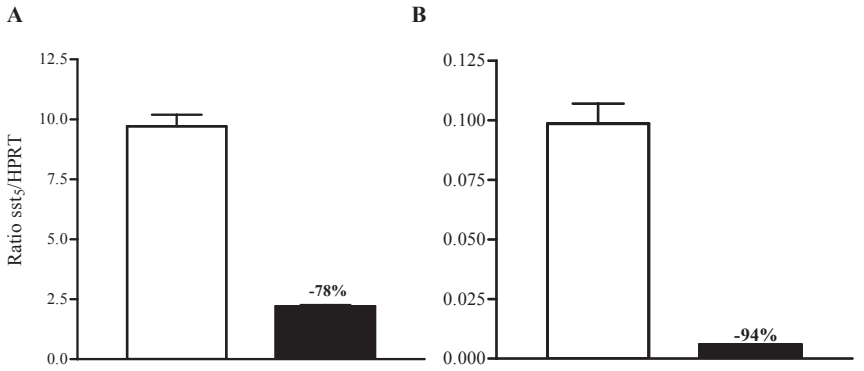

Figure 3. Quantification of sst_5 gene expression and analysis of sst_5 receptor mRNA in transiently (72 hr) co-transfected HEK 293 cells with (A) 5µg sst_5 or (B) 0.5µg sst_5 plasmid and 5µg siRNA plasmid. Sst_5 and HPRT mRNA expression levels were quantified by a TaqMan assay and results are depicted as bars, representing the means ± S.E. from two experiments performed in quadruplicate. Control group is transfected with 5µg pSuper vector. ■, siRNA ; □, control.

Discussion

Over the past years RNAi has been introduced as a new approach to manipulate gene expression in mammalian systems. On the basis of a new mammalian expression vector that directs the synthesis of short hairpin siRNA-like transcripts [pSUPER] (6), which have been demonstrated to trigger gene silencing (22, 23), we synthesized a siRNA constructs for sst_2 and sst_5 and evaluated its efficacy to down-regulate sst gene expression. Effective silencing of the sst_2 and sst_5 mRNA expression in sst-transfected CHO-K1 cells and HEK 293 cells, respectively, by use of our RNAi constructs was successful. It is generally believed that the siRNA needs to be double stranded in order to be efficiently recognized and bound to RISC, the two siRNA strands must unwind before RISC becomes active (24). Several studies revealed that only the antisense strand of the siRNA is incorporated into the enzymatic machinery of the RISC complex and effective siRNA exhibited decreased stability of the 5' strand of the antisense strand (18, 25, 26). It should therefore be desirable to design the siRNA duplex in a way that the antisense strand is the one to be preferentially used by RISC. This prompted us to synthesize two additional sst_2 targeted siRNA constructs (H-523 and H-573) which enrolled these guidelines (18).

Thermodynamic profiles, the standard Gibbs free energies, which reflect the stability of pentamer sequences, would favour the H-523 construct as being efficacious to silence the sst_2 gene as it contains a rich A/T region at the 5' end of the antisense strand. However, H-523 was not effective to down-regulate sst_2 mRNA expression in our model, while the other two constructs suppressed sst_2 expression with approximately 75%. It should be noted that highly effective siRNA sequences can be found that do not adhere these criteria and that some siRNAs that adhere to the criteria do not function well (26). Interestingly, in retrospect, the synthesized siRNA-sst_5 construct appeared to follow several of these guidelines, i.e. rich A/T area at 5' of the antisense strand, a rich C/G area at the 5' end of the sense strand and the absence of any GC stretch of more than 9nt in length (18). A vital assumption in the application of siRNA-mediated RNAi as a functional genomics tool is that the knockdown of a targeted gene is specific both at the RNA and protein level. Additional experiments will have to be done to demonstrate that the siRNA constructs for sst we have developed are also capable to reduce the function of the receptor as well. Studies that involve specific sst-membrane binding with the use of radiolabeled SRIF-14 or studies which evaluate SRIF-induced inhibition of adenylate cyclase activity, seem of interest for future experiments. In conclusion, we have demonstrated that the siRNA constructs for the sst_2 and sst_5 gene we have synthesized merit to become novel tools to eventually evaluate the individual role of sst_2 and sst_5 receptors endogenously expressed in primary cells, such as GH-secreting pituitary adenomas. At present, the transfectability of cells is one of the limiting steps in siRNA-mediated gene silencing, especially in primary cells which are notorious for having low transfection efficiencies when plasmid DNA is used. Furthermore, several aspects of RNAi, i.e. non-specific silencing effects (27) and activation of the interferon response (4) may occur when endogenous (sst-) expressing mammalian cell systems are used. As of now, it is not clear how often siRNAs trigger the interferon pathway and which conditions favour this response to these RNAs. Chemical features of dsRNAs, as well as their expression levels and delivery routes, may determine whether they become visible to the interferon response machinery (4, 28).

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Chapter V

Somatostatin Receptor Subtype 5 in Cushing's Disease; in vitro Support for Novel Therapeutic Opportunities

Chapter V-1

The multiligand somatostatin analog SOM230 inhibits ACTH secretion by cultured human corticotroph adenomas via somatostatin receptor subtype 5

Eur J Endocrinol. 2005, 152:645-54

Abstract

Objective: Currently, there is no effective medical treatment in patients with pituitary dependent Cushing's disease. A novel somatostatin (SRIF)-analog, named SOM230, with high binding affinity to sst_1, sst_2, sst_3, and sst_5 was recently introduced. We compared the in vitro effects of the sst_2-preferring SS-analog octreotide (OCT) and the multiligand SOM230 on ACTH release by human and mouse corticotroph tumor cells.
Methods: By quantitative RT-PCR the sst subtype expression level was determined in human corticotroph adenomas. In vitro, the inhibitory effect of OCT and SOM230 on ACTH release by dispersed human corticotroph adenoma cells and mouse AtT20 corticotroph adenoma cells was determined. In addition, the influence of dexamethasone on the responsiveness to OCT and SOM230 was studied.
Results: Corticotroph adenomas expressed predominantly sst_5 mRNA (6/6 adenomas), whereas sst_2 mRNA expression was detected at significantly lower levels. In a 72 h incubation with 10nM SOM230, ACTH release was inhibited in 3 out of 5 cultures (range -30 to -40%). 10nM OCT slightly inhibited ACTH release in only 1 of 5 cultures (-28%). In AtT20 cells, expressing sst_2, sst_3 and sst_5, SOM230 inhibited ACTH secretion with high potency (IC_{50};0.2nM). Dexamethasone (10nM) pre-treatment did not influence the sensitivity of the cells to the inhibitory effect of SOM230, suggesting that sst_5 is relatively resistant to negative control by glucocorticoids.
Conclusions: The selective expression of sst_5 receptors in corticotroph adenomas and the preferential inhibition of ACTH release by human corticotroph adenoma cells by SOM230 in vitro, suggest that SOM230 may have potential in the treatment of patients with pituitary dependent Cushing's disease.

Introduction

The first choice of treatment of patients with pituitary-dependent Cushing's disease is surgery. If surgery fails, radiotherapy, alone or in combination with steroidogenic inhibitors, may be used (1, 2). These secondary options are primarily due by the absence of an effective medical treatment option (3, 4). Some preliminary data suggest a potential use of dopamine (DA) agonists, alone or in combination with ketoconazole, in selected cases of Cushing's disease or Nelson's syndrome (2). The expression of somatostatin (SRIF) receptors (sst) in adrenocorticotropin

(ACTH)-secreting pituitary adenomas has been studied in vivo and in vitro. In vivo, none of a series of eight corticotroph microadenomas showed an increased uptake of ^{111}In-DTPA-octreotide, whereas sst scintigraphy was positive in two invasive ACTH-secreting macroadenomas and two cases of Nelson's tumors (5). The current generation of sst_2-preferential SRIF-analogs have no suppressive effect on ACTH level in patients with untreated Cushing's disease, who have elevated cortisol levels (6). However, the SRIF-analog octreotide (OCT) suppressed pathological ACTH release in patients with Nelson's syndrome and ACTH and cortisol secretion in patients with Cushing's syndrome caused by ectopic ACTH secretion (7-9). Overall, these data suggest that in corticotroph adenomas of untreated patients with Cushing's disease sst_2 levels, one of the five known sst subtypes to which OCT binds preferentially, are low, and that this receptor subtype may be down-regulated when circulating cortisol levels are high. The fact that ectopic ACTH secreting tumours express sufficient sst numbers allowing their in vivo visualization by sst scintigraphy and responsiveness to octreotide, even in the presence of a high cortisol level, can be explained by their relative resistance to glucocorticoids, compared with corticotroph adenomas (10). Additional in vitro evidence for a sst downregulation by hypercortisolaemia comes from studies using primary cultures of human corticotroph adenomas, in which it was shown that glucocorticoids down-regulated the response of corticotrophin releasing hormone (CRH)-induced ACTH secretion to octreotide (11). Little is know yet about the quantitative expression levels of the five sst subtypes (sst_{1-5}) in normal and tumoral corticotroph cells. By double labelling in situ hybridization analysis for sst_2 and sst_5 mRNA, Day et al. (12) showed that normal rat corticotrophs expressed preferentially sst_5 mRNA. In a few selected cases expression of sst subtype mRNA transcripts was studied in human corticotroph adenomas. In these studies it was found that the highest frequency of expression of sst mRNA transcripts was found for sst_2 and sst_5, and to a lesser extent sst_1 (13-16). Although sst_2 mRNAs can be found in corticotroph adenomas, their levels of expression are apparently low since octreotide has no effect on ACTH secretion in patients with pituitary dependent Cushing's disease.

However, at present the role of the other frequently expressed sst subtype, sst_5, on ACTH secretion by corticotroph adenoma cells is unclear. Recently, a novel multiligand SRIF-analog SOM230 has been synthesized. Compared with octreotide, SOM230 has a 30-, 5- and 40-times higher binding affinity to sst_1, sst_3 and sst_5 receptors, respectively, and 2.5 times lower affinity to sst_2 (17). The very high affinity of this SRIF-analog for sst_5 probably forms the basis for the higher potency of SOM230 in reducing IGF-I concentrations in rats, primates and dogs (18). Moreover, the very favourable elimination half-life of SOM230, which is 23 h (17), makes this novel compound suitable for clinical application as well. The present study was carried out to further explore the potential functional significance of sst subtypes expressed in human corticotroph adenomas. We evaluated the effect of the SRIF-analog SOM230 on ACTH secretion by primary cultures of human corticotroph adenomas and mouse AtT20 corticotroph tumor cells.

Materials and Methods

Patients

Pituitary tumor samples were obtained by transsphenoidal operation from 11 patients with Cushing's disease due a corticotroph adenoma as described in detail previously (19). ACTH-dependent hypercortisolism was biochemically established by the absence of cortisol diurnal rhythm, an increased 24 h free cortisol excretion, insufficient suppression of plasma cortisol concentrations after administration of 1 mg of dexamethasone and normal to increased plasma ACTH concentrations. MRI of the pituitary showed a microadenoma in 7 patients, a macroadenoma in 1 patient and no adenoma in 3 patients. In these latter patients pituitary ACTH overproduction was determined by sinus petrosus inferior sampling. Histological examination showed adenomatous tissue (n=9; case no. 1 and no.'s 4-11) or hyperplasia (n=2; case no.'s 2 and 3) with immunohistochemically expression of ACTH. All patients gave their informed consent for the use of tumor material for research purposes. After surgery, 7 of 11 patients were cured. In six cases (no. 1-6) the tissue was directly snap-frozen on dry ice

and stored at -80 C until analysis. Adenoma tissue from the 5 additional patients (culture no. 7-11) was directly used for cell culturing.

Quantitative PCR

Quantitative PCR was performed as described previously (20). Briefly, poly A^+ mRNA was isolated from adenoma tissue using Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway). cDNA was synthesized using the poly A^+ mRNA, which was eluted from the beads in 40 µl H_2O for 2 min at 65 °C, using Oligo $(dT)_{12\text{-}18}$ Primer (In Vitrogen, Breda, The Netherlands). One-twentieth of the cDNA library was used for quantification of sst subtype mRNA levels. A quantitative PCR was performed by TaqMan® Gold nuclease assay (Perkin Elmer Corporation, Foster City, CA, USA) and the ABI PRISM® 7700 sequence Detection System (Perkin Elmer, The Netherlands) for real-time amplifications, according to manufacturer's protocol. The assay was performed using 15µl TaqMan® Universal PCR Master Mix (Applied Biosystems, The Netherlands), 500 nM forward primer, 500 nM reverse primer, 100 nM probe and 10µl cDNA template, in a total reaction volume of 25 µl. After an initial heating at 95° C for 8 min, samples were subjected to 40 cycles of denaturation at 95° C for 15 seconds and annealing for 1 min at 60° C. The primer and probe sequences that were used are indicated below. The detection of hypoxantine-phosphoribosyl-transferase (hprt) mRNA served as a control and was used for normalisation of the sst subtype mRNA levels. The primer and probe sequences that were used for the detection of sst_1, sst_2, sst_3, sst_5 and hprt mRNA's have been described previously (20). In addition to these primers and probes, we also evaluated sst_4 mRNA expression in the present study using the following primers and probe: **Sst_4** forward 5'-CTGCGCCAACCCTATTCTCT-3'; **Sst_4** reverse 5'-ACCCGCTGGAAGGATCG-3'; **Sst_4** probe 5'-FAM-TGGCTTCCTCTCCGACAACTTCCG-TAMRA-3'. Primers and probes were purchased from Biosource (Nivelles, Belgium). The relative amount of sst subtype mRNA was determined using a standard curve generated from known amounts of human ge-

nomic DNA. For determination of the amount of HPRT mRNA, a standard curve was generated of a pool of cDNAs from a human cell line known to express HPRT. The relative amount of sst subtype mRNA was calculated relative to the amount of HPRT mRNA and is given in arbitrary units. Each sample was assayed in duplicate. Poly A^+ mRNA from AtT20/D16V cells was isolated as described above. PCR analysis to determine the expression of mouse sst_{1-5} mRNAs was performed as described elsewhere (21).

Cell dispersion and cell culture

Pituitary adenoma tissue

Single cell suspensions of the pituitary adenoma tissues were prepared by enzymatic dissociation with dispase as described in detail previously (19). For short-term incubation of monolayer cultures, the dissociated cells were plated in 48-well plates (Corning BV Life Sciences, Schiphol-Rijk, The Netherlands) at a density between 10,000 to 50,000 cells per well per 1 ml culture medium. After 3 - 4 days the medium was changed and 4, 24 or 72 h incubations without or with test-substances were initiated. At the end of the incubation the medium was collected and stored at –20 C until hormone determination. The cells were cultured at 37 C in a CO_2-incubator. The culture medium consisted of Minimum Essential Medium with Earle's salts (MEM) supplemented with non essential amino acids, sodium pyruvate (1 mmol/L), 10% fetal calf serum (FCS), penicillin ($1x10^5$ U/L), fungizone (0.5 mg/L), L-glutamine (2mmol/L), and sodium bicarbonate (2.2 g/L), pH 7.6. Media and supplements were obtained from In Vitrogen (Breda, The Netherlands). Unfortunately, not enough tumor material was obtained to test the adenoma cells at all timepoints and on the dose-dependency of the effects by the indicated drugs.

Mouse corticotroph adenoma cells

AtT20/D16V mouse corticotroph tumor cells (Dr. J. Tooze; European Organization Molecular Biology) were routinely passaged by trypsinization as described in detail previously (22). The cells were main-

tained in 75 cm^2 culture flasks in Dulbecco's Minimal Essential Medium (DMEM), supplemented with non essential amino acids, sodium pyruvate (1 mmol/L), 10% fetal calf serum (FCS), penicillin ($1x10^5$ U/L), fungizone (0.5 mg/L), L-glutamine (2mmol/L), and sodium bicarbonate (2.2 g/L), pH 7.6. For experiments, the cells were seeded at a density of 20,000 cells per well in 1 ml of culture medium. After 72 h, the medium was changed and 4, 24 or 72 h incubations without or with OCT, SOM230 or SRIF were initiated. In addition, the acute effect of SRIF, OCT and SOM230 was evaluated in a 3h incubation without or with 10nM CRH. In order to evaluate the effect of glucocorticoids on SRIF-analog induced inhibition of ACTH release, the cells were pre-treated in some experiments for 48h with the synthetic glucocorticoid dexamethasone (10nM). Thereafter, the medium was changed and a 3 h incubation without or with octreotide (10nM) or SOM230 (10nM) in the presence of CRH (10nM) was performed. After the indicated time periods, the medium was collected and stored at -20^0C until determination of ACTH concentrations. For determining the effects of the compounds on cell growth, ^{3}H-thymidine incorporation, as well as the DNA content of the wells, was measured as described in detail elsewhere (23).

Hormone determinations

Human ACTH concentrations were determined by a non-isotopic, automatic chemiluminescence immunoassay system (Immulite, DPC Inc.). Intra- and interassay CV's were 5.6% and 7.8%, respectively. Human GH, PRL, LH and FSH concentrations in the media were determined, in order to exclude the presence of contaminating normal pituitary cells in the cultures. GH, PRL, LH and FSH concentrations were determined by a non-isotopic, automatic chemiluminescence immunoassay system as well (Immulite, DPC Inc., Los Angeles, CA). Intra- and interassay CV's for GH, PRL, LH and FSH were 6.0%, 5.7%, 5.7%, 6.4% and 6.2%, 6.4%, 12.3%, 7.5%, respectively. Except for the expected hormone ACTH, none of the other hormones were detectable (not shown).

Test-substances

Octreotide (OCT; Sandostatin®) was obtained from Novartis Pharma A.G. (Basle, Switzerland). SOM230 was a gift from Novartis Pharma A.G. Somatostatin-14 (SRIF) was purchased from Sigma Chemical Co. (St. Louis, MO). Binding affinities of SRIF, OCT and SOM230 to the five sst are shown in table I. Dexamethason was obtained from the Erasmus MC Pharmacy (Rotterdam, The Netherlands). Corticotropin-releasing hormone (CRH) was purchased from Ferring (Hoofddorp, The Netherlands).

Table I. Binding affinity of SRIF, OCT and SOM230 for $sst_{1\text{-}5.}$

Compound	*hsst$_1$*	*hsst$_2$*	*hsst$_3$*	*hsst$_4$*	*hsst$_5$*
SRIF	0.93 ± 0.12	0.15 ± 0.02	0.56 ± 0.17	1.5 ± 0.4	0.29 ± 0.04
OCT	280 ± 80	0.38 ± 0.08	7.1 ± 1.4	>1000	6.3 ± 1.0
SOM230	9.3 ± 0.1	1.0 ± 0.1	1.5 ± 0.3	>100	0.16 ± 0.01

Data are reproduced with permission (17). Results are the mean ± SE, IC_{50} values are expressed in nM.

Statistical analysis of the data

All data on hormone release are expressed in mean ± SE, n=4 wells per treatment group. All data were analyzed by analysis of variance (ANOVA) to determine overall differences between treatment groups. When significant differences were found by ANOVA, a multiple comparison between treatment groups was made using the Newman-Keuls test. Calculation of IC_{50} values for inhibition of hormone release were made using GraphPad Prism (San Diego, CA).

Results

Somatostatin receptor subtype mRNA expression

In 6 out 6 human corticotroph adenomas (no's 1-6), sst_2 mRNA was detectable. However, the relative copy numbers were low (varying between 8 and 141 copies/hprt). The relative sst_2 copy number was considerably lower compared with the majority of GH-secreting pituitary

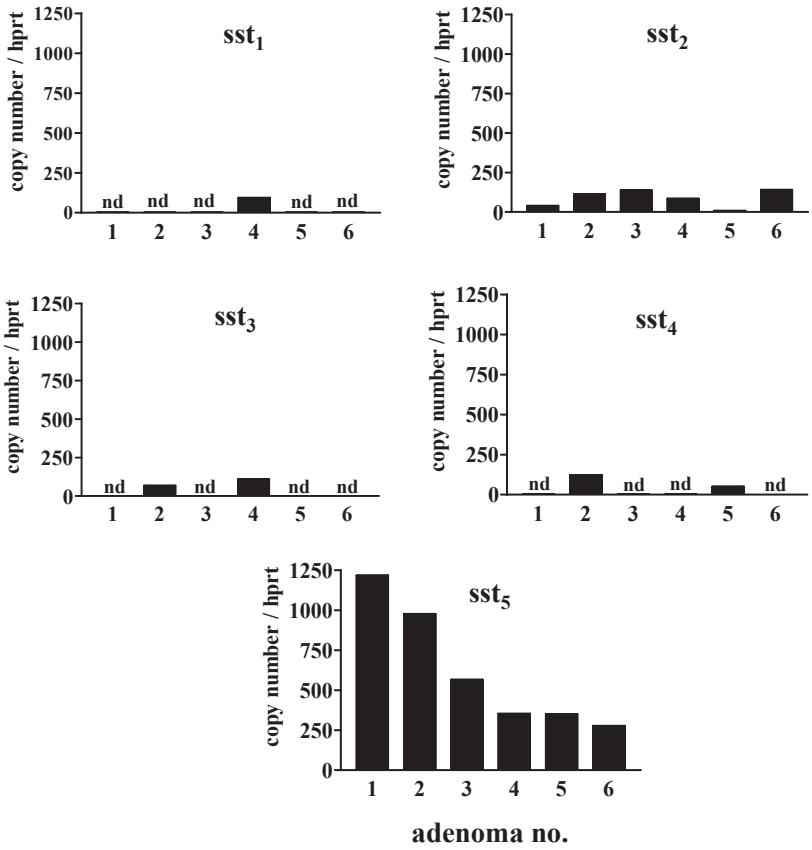

Figure 1. SRIF receptor subtype mRNA expression level in human corticotroph adenoma. mRNA levels were determined by real time PCR of cDNA obtained from six human corticotroph adenomas (no. 1-6). Values are expressed as the number of copies relative to hprt mRNA.

adenomas, as recently reported (20). Sst_5 mRNA was detectable at much higher levels in 6/6 adenomas (relative copy number between 277-1217). In the two cases in which histological analysis of the tissue revealed hyperplasia of ACTH-producing cells (case no.'s 2 and 3), sst_5 mRNA level were relatively high. On the other hand, one ACTH-secreting pituitary adenoma (case no. 1), expressed the highest level of sst_5 mRNA in the series. Sst_1, sst_3 and sst_4 mRNA was detectable at low levels in only 1/6, 2/6 and 2/6 samples, respectively. This is shown in figure 1. In agreement with the results of RT-PCR analysis of the corticotroph adenomas, the sst subtype mRNAs expressed in ACTH-secreting mouse AtT20 cor-

ticotroph pituitary tumor cells were sst_2, sst_3 (low abundance) and sst_5 (fig. 2).

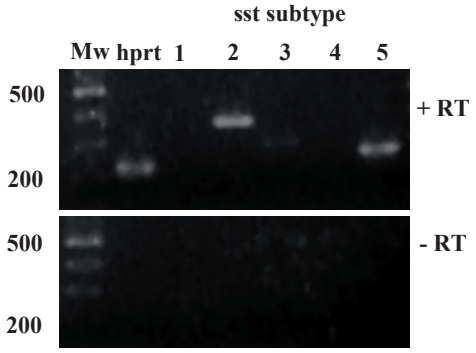

Figure 2. Expression of sst subtypes in mouse AtT20 pituitary tumor cells. Poly A+ mRNA was reverse transcribed and cDNA was amplified by PCR. PCR products of the sst_{1-5} were separated on 1% agarose gel and stained with ethidium bromide. Left panel represents cDNA synthesis in the presence of reverse transcriptase (+RT); right panel represents negative control of cDNA synthesis in the absence of RT (-RT) to exclude the presence of genomic DNA contamination. Only bands of sst_2, sst_3 and sst_5 PCR products with the expected molecular weight (Mw) were detected. hprt: house-hold keeping gene hypoxantine-phosphoribosyl-transferase.

Effect of SRIF, octreotide and SOM230 on basal and CRH-induced ACTH secretion by primary corticotroph adenoma cells

Since not sufficient tissue was obtained to carry out both mRNA- and culture studies on the same tissue, cell culture experiments were performed using adenoma tissue from 5 additional patients (no.'s 7-11). In a 72 h incubation, SOM230 (10nM) significantly suppressed ACTH secretion in 3/5 primary cultures of human corticotroph adenomas (between 30 and 40% suppression). In contrast, OCT (10nM) suppressed basal ACTH release in only 1/5 cultures (28% suppression). This is shown in figure 3A and 3B. From one corticotroph adenoma (no.8) sufficient cells were obtained to perform a time course study of the effects of SRIF, OCT and SOM230 on basal ACTH release. As shown in figure 4A (4, 24 and 72 h incubation), statistically significant ($P<0.01$ vs untreated control cells) suppression of ACTH release was observed only after 72 h of incubation with SOM230 (10nM) and SRIF (10nM). 10nM OCT did not significantly inhibit basal ACTH release at any time point. A comparable time-course experiment

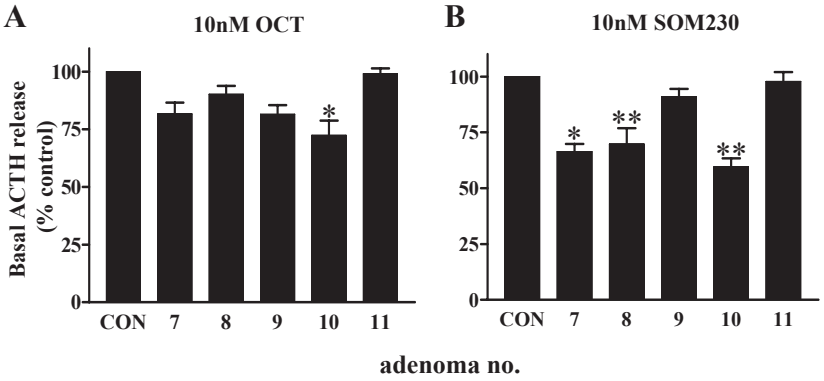

Figure 3. Effect of 10nM OCT (A) and 10nM SOM230 (B) on basal ACTH release from five cultured human corticotroph adenomas (no. 7-11). The cells were incubated in the absence or presence of octreotide (panel A) or SOM230 (panel B) during 72 h after which the medium was collected for ACTH determination. Values are expressed as % of control (CON; untreated cells). Basal ACTH concentrations in the culture media were 239 ± 5.0, 28800 ± 1159, 25100 ± 2150, 218 ± 12 and 3228 ± 153 fmoles/well, for cultured adenomas no. 7, 8, 9, 10 and 11, respectively. *p<0.05 and **p<0.01 vs control.

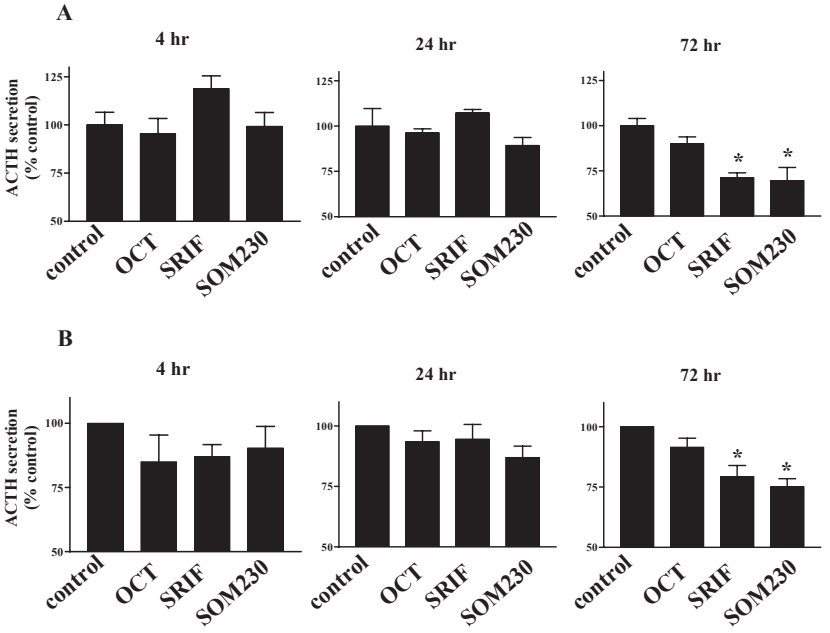

Figure 4. Time-dependent effect of OCT, SOM230 and SRIF (10 nM) on basal ACTH release from cultured human corticotroph adenoma no.8 (A) and mouse AtT20 tumor (B) cells. The cells were incubated for 4, 24 or 72 h in the absence or presence of the indicated compounds after which medium was collected for ACTH determination. Values are expressed as the % of ACTH release by control (untreated) cells at the indicated time-points. For the human corticotroph adenoma cells, basal ACTH values were 3505 ± 232 (4 h), 9778 ± 946 (24 h) and 28800 ± 1159 (72 h) fmoles/well. *p<0.01 vs control.

was performed in mouse AtT20 cells. In agreement with the observations in primary human corticotroph adenoma cultures, SOM230 and SRIF, but not OCT, all tested at 10nM, suppressed basal ACTH production (fig. 4B). The effect of SOM230 on basal ACTH release by AtT20 cells was dose-dependent with an IC_{50} value of 0.2 nM (fig. 5A), corresponding to

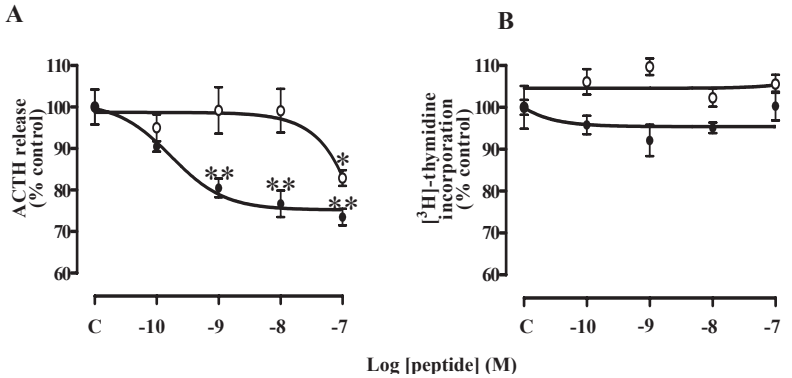

Figure 5. Dose-dependent effect of SOM230 and OCT on basal ACTH release (panel A) and cell proliferation as measured by [^{3}H]-thymidine incorporation (panel B) by mouse AtT20 pituitary tumor cells. AtT20 cells were incubated during 72h without or with increasing concentrations of OCT (open circles) or SOM230 (closed circles) after which the medium was collected for ACTH determination. Values are expressed as the % of control (untreated) cells. *p<0.05 and **p<0.01 vs control.

the binding affinity of SOM230 for sst_5. In contrast, OCT inhibited ACTH release with much lower potency with a slight suppression at 100nM (fig. 5A), which did not reach statistical significance, however. Taken together, these data suggest that the effect of SOM230 on basal ACTH release is most likely mediated via sst_5. OCT and SOM230 did not inhibit AtT20 cell proliferation as measured by ^{3}H-thymidine incorporation (fig. 5B), and had no effect on the DNA content of the cells (not shown). In contrast to the absence of an effect of OCT on basal ACTH release by AtT20 cells, OCT (10nM) significantly suppressed CRH (10nM)-induced ACTH release by 52% and SRIF by 47% in a 3 h incubation. SOM230 was significantly more potent and inhibited CRH-induced ACTH release by 76% (fig. 6). The effect of SRIF on CRH-induced ACTH-release by AtT20 cells is in agreement with previous reports (24-26).

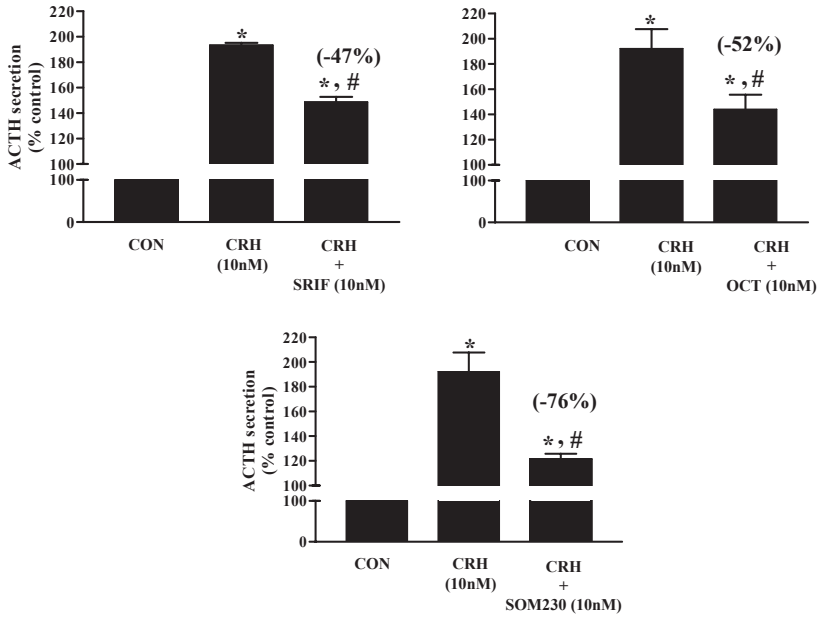

Figure 6. Effect of OCT, SRIF and SOM230 on CRH-stimulated ACTH release by mouse AtT20 pituitary tumor cells. AtT20 cells were incubated for 3 h in the absence or presence of CRH and SRIF, OCT or SOM230 after which the medium was collected for ACTH determination. Values are expressed as the % of control (untreated) cells. *p<0.01 vs control, #p<0.01 vs CRH alone.

Effect of glucocorticoid pre-treatment

In order to evaluate whether pre-treatment of the cells with glucocorticoids influenced the sensitivity to the effects of OCT and SOM230, mouse AtT20 cells were pre-treated with 10 nM dexamethasone (DEX) during 48 h, after which a 3 h incubation without or with CRH (10 nM), OCT (1 nM) and/or SOM230 (1 nM) was performed. Figure 7 shows that pre-treatment of AtT20 cells with 10 nM DEX had no effect on the inhibitory effect of SOM230 on CRH-induced ACTH release. On the other hand, the inhibitory effect of 1 nM OCT was completely abolished by Dex pre-treatment. In the presence of Dex, SOM230 almost completely abolished CRH-induced ACTH release (fig. 7, lower panel). Finally, Dex-pretreatment did not change the inhibitory effects of 10nM OCT or SOM230 on CRH-induced ACTH release (data not shown), suggesting that at OCT at 10nM, induced its inhibitory effect via interaction with sst_5., to which it

has significant lower affinity compared with SOM230 (Table I). Moreover, in one primary culture of corticotroph adenoma cells (case no. 4), which was unresponsive to OCT, DEX (10nM) pre-treatment did not reduce the inhibitory effect by SOM230 on CRH (10 nM)-induced ACTH release as well (-21% without DEX vs -33% in the presence of DEX).

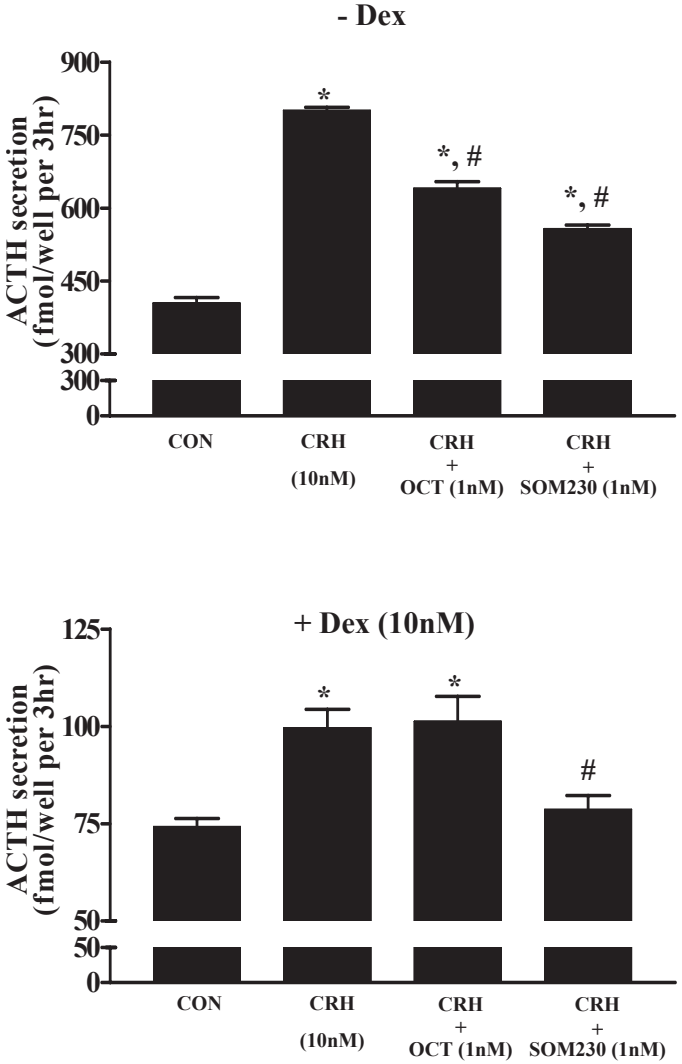

Figure 7. Effect of glucocorticoids on OCT and SOM230 mediated inhibition of CRH-stimulated ACTH release by mouse AtT20 pituitary adenoma cells. AtT20 cells were pre-incubated during 48 h without or with 10nM dexamethasone (Dex). After 48 h, the medium was refreshed and the cells were incubated for 3 h in the absence or presence of Dex, CRH (10nM) and OCT (1 nM) or SOM230 (1 nM) after which the medium was collected for ACTH determination. *$p<0.01$ vs control, #$p<0.01$ vs CRH alone.

Discussion

Currently, there is no effective medical treatment option in patients with pituitary-dependent Cushing's disease (3, 4). Therefore, in the present study we evaluated the effects of the recently developed multiligand stable SRIF-analog SOM230 (17, 18, 27), which has high binding affinity to sst_1, sst_2, sst_3 and sst_5 on ACTH release by human corticotroph tumors in vitro. The role of SRIF in the regulation of normal ACTH release is equivocal. Previous in vitro studies showed no inhibitory effect of SRIF on basal- and CRH-induced ACTH release by normal rat anterior pituitary cells (28) and perifused normal rat pituitary halves (29). On the other hand, SRIF inhibited CRH-stimulated ACTH release by fragments of pituitary glands (30), as well as arginine vasopressin-induced ACTH release by cultured pituitary cells from long-term adrenalectomized rats (31). The latter study suggests that normal corticotrophs only respond to SS in the presence of very low cortisol concentrations. Confirming this hypothesis, we demonstrated that SRIF inhibits CRH-stimulated ACTH secretion by normal rat anterior pituitary cells only when the cells are cultured in the absence of glucocorticoids in the culture medium (32). In addition, pre-incubation of the cells with the progesterone-glucocorticoid receptor-blocking compound RU 38486, increased the sensitivity of ACTH secretion to the inhibitory effect by SS and pre-treatment with dexamethasone made the cells insensitive to SRIF (32). The high levels of cortisol in patients with pituitary dependent Cushing's disease can thus be responsible for the observed lack of inhibition of ACTH release by SRIF and/or OCT in these patients (6, 11, 33). Moreover, in patients with Nelson syndrome and adrenal insufficiency of different origin, SRIF and/or OCT lower ACTH secretion (6, 9, 34, 35). In cultured corticotroph adenomas, SRIF and/or OCT inhibit ACTH secretion in part of the cultures (11, 36-38). Stalla et al. (11) showed that hydrocortisone treatment in vitro abolished the inhibitory effect of octreotide on ACTH secretion, possibly due to a down-regulation of sst on the corticotrophs. In agreement with this hypothesis, cortisol reduced [^{125}I-Tyr^1]SRIF binding on mouse AtT20/D16 pituitary tumor cells by 20-40% (39), although the subtype sst was not characterized.

Little is known with respect to sst subtype expression in pituitary corticotrophs. In rat pituitary cells, co-localization of all five sst with ACTH expressing cells has been reported (40). In another study, sst_5 mRNA was found in 38% of normal corticotrophs, and sst_2 in only 3%. Moreover, the average number of grains per cell was also higher for sst_5 than sst_2 (12). This suggests that under normal physiological conditions, sst_5 is predominantly more expressed in rat corticotrophs than sst_2. As far as known, no data on the quantitative expression of sst mRNA in human corticotroph adenomas are available. In the present study we found that at the mRNA level, sst_5 was the predominantly expressed receptor type in human corticotroph adenomas. In the small series of tissues (n=6) analyzed in this study, two cases of hyperplasia of corticotroph cells, expressed a relatively high sst_5 mRNA level. On the other hand, one corticotroph adenoma expressed the highest sst_5 mRNA level in this series. The number of cases is too low, however, to establish whether there is a difference in sst_5 mRNA expression between hyperplasia of ACTH-producing cells and ACTH-secreting adenoma. Sst_2 mRNA was expressed in all cases as well, although at a much lower level. The high frequency of expression of sst_2 and sst_5 in corticotroph adenomas is well in agreement with other reports. mRNA expression in human corticotroph tumors shows the overall presence of sst_1 in 5/8 (63%), sst_2 in 7/8 (88%), sst_3 in 3/9 (33%), sst_4 in 1/8 (12%), and sst_5 in 6/7 (86%) cases (13-15, 41, 42). The low expression levels of sst_2 may explain the lack of efficacy of the sst_2-preferring agonist OCT in lowering circulating ACTH and cortisol levels in untreated patients with pituitary-dependent Cushing's disease (6, 11). In vitro we found a statistically significant inhibition of basal ACTH release by OCT in only 1/5 cases. The higher number of cultures (3/5) responding to a maximally active concentration of 10 nM SOM230, which has very high binding affinity to sst_5, is in agreement with the higher expression levels of sst_5 mRNA. Unfortunately, not enough tissue was obtained to perform both mRNA analysis and cell culture studies. In one adenoma, which had an intermediate sst_5 mRNA level (case no. 4), SOM230 already inhibited ACTH secretion, whereas OCT had no effect. It should be noted that only mRNA levels were studied. Future

studies should demonstrate whether sst_5 receptors are expressed in corticotroph adenomas at a high protein level as well. The importance of sst_5 in regulating ACTH release was further confirmed by our observation that SOM230 was also much more potent compared with OCT in inhibiting basal ACTH release by mouse AtT20/D16V corticotroph tumor cells. The pattern of inhibition, e.g. a higher potency of SOM230 vs OCT, is in line with the sst binding profile of both SRIF-analogs. Recently, Cervia et al. (43) showed that sst_2 is the predominant functional receptor subtype in AtT20 cells, while sst_5 is also able to mediate inhibition of ACTH release when the ligand is not able to activate sst_2 receptors. Interestingly, while SOM230 was much more potent in inhibiting basal ACTH release by AtT20 cells, we found that OCT also potently inhibited CRH-induced ACTH release. However, maximal inhibition of ACTH by SOM230 was significantly higher compared with OCT. These data suggest that expression of sst_2 in our AtT20 line is relatively low, compared with sst_5. The involvement of both sst_2 and sst_5 in the regulation of ACTH release is further underlined by the observation that sst_2- and sst_5-specific agonists potently inhibit ACTH release and cAMP production by AtT20 cells (44). As indicated above, glucocorticoids may down-regulate sst on AtT20 corticotrophs and lower responsiveness of ACTH to the inhibitory effect of SRIF and/or octreotide. Therefore, we also studied whether pre-treatment of the cells with the synthetic glucocorticoid dexamethasone (10nM) reduced responsiveness of the cells to inhibition of ACTH release by SOM230. We found that glucocorticoid pre-treatment did not influence the inhibitory effect of SOM230 on CRH-induced ACTH release, suggesting that the expression of functional sst_5 receptors is relatively resistant to glucocorticoids. This may also explain the higher expression levels of sst_5 mRNA we found in human corticotroph adenomas from untreated patients with pituitary-dependent Cushing's disease. These data suggest that glucocorticoids may have differential regulatory effects on sst_2 and sst_5 expression, respectively. An intriguing observation is that SOM230 and SRIF only inhibit basal ACTH secretion after prolonged in vitro exposure. SOM230 did not inhibit AtT20 cell proliferation. Therefore, other mechanisms, e.g. inhibition of POMC synthesis and/or increased ACTH

breakdown, as has been shown for the effect of OCT on GH-secreting pituitary adenomas (45, 46) may form additional explanations for the inhibitory of SOM230 and SRIF on basal ACTH secretion. Interestingly, recent evidence suggests that sst_5 is also important in the control of ACTH secretion in vivo. Sst_5 knock out mice were shown to have significantly elevated ACTH and corticosterone levels, compared with wild type mice (47). While our data suggest that the novel SRIF-analog SOM230 may be useful in the medical management of patients with pituitary-dependent Cushing's disease, several issues will have to be clarified. First, prolonged treatment of AtT20 cells with SRIF results in desensitization of its inhibitory effect on ACTH secretion and cAMP formation (48, 49). In addition, prolonged agonist exposure of AtT20 cells with SRIF-14 or SRIF-28 was shown to down-regulate sst receptors (50). However, these studies only evaluated the acute inhibitory effects of SRIF on AtT20 cells. Moreover, the effects of SOM230 are most likely sst_5-mediated and sst_5 receptors have shown to be rapidly recycled and recruited from intracellular storages after agonist activation. This combination of recycling and recruitment of spare sst_5 receptors may protect from long-term down-regulation through sequestration and, therefore, facilitate extended SRIF-signaling (51). We observed that the inhibitory effects of SOM230 on basal ACTH secretion became only apparent after prolonged exposure. This observation already suggests that endogenously expressed sst_5 receptors may be more resistant to desensitization and/or downregulation. Nevertheless, the in vivo effect of SOM230 on ACTH secretion by corticotroph adenomas needs to be further evaluated. In conclusion, the selective expression of sst_5 receptors in human corticotroph adenomas, in combination with the inhibitory effect of SOM230 on basal and CRH-induced ACTH secretion, suggest that SOM230 may have potential in the medical treatment of pituitary-dependent Cushing's disease.

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Chapter V-2

Distinct functional properties of native somatostatin receptor subtype 5 compared with subtype 2 in the regulation of ACTH release by cultured mouse corticotroph tumour cells

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Abstract

In a series of human corticotroph adenomas, we recently found predominant mRNA-expression of somatostatin (SRIF) receptor subtype (sst) 5. After 72h, the multiligand SRIF-analog, SOM230, which has a very high sst_5 binding affinity, but not Octreotide (OCT), significantly inhibited basal ACTH release. To further explore the role of sst_5 in the regulation of ACTH release, we conducted additional studies with mouse AtT-20 cells. SOM230 showed a 7-fold higher ligand binding affinity and a 19-fold higher potency in stimulating GTPγS binding in AtT-20 cell membranes as compared with OCT. SOM230 potently suppressed CRH-induced ACTH release, which was not affected by 48h dexamethasone (DEX) pre-treatment. However, DEX attenuated the inhibitory effects of OCT on ACTH release, whereas it increased the inhibitory potency of BIM-23268, a sst_5-specific analog, on ACTH release. Quantitative-PCR analysis showed that DEX lowered sst_{2A+2B} mRNA expression significantly after 24 and 48h, whereas sst_5 mRNA levels were not significantly affected by DEX treatment. Moreover, Scatchard analyses showed that DEX suppressed maximum binding capacity (Bmax) by 72% when [^{125}I-Tyr^3]-Octreotide was used as radioligand, whereas Bmax declined only by 17% when AtT-20 cells were treated with [^{125}I-Tyr^{11}]-SRIF-14. These data suggest that the sst_5 protein, compared with sst_2, is more resistant to glucocorticoids. Finally, after SRIF-analog pre-incubation, compared with OCT, both SOM230 and BIM-23268 showed a significant higher inhibitory effect on CRH-induced ACTH release. In conclusion our data support the concept that the sst_5 receptor might be a target for new therapeutic agents to treat Cushing disease.

Introduction

Cushing's disease, the pituitary-dependent form of Cushing's syndrome, is the hypercortisolemic state secondary to excess or dysregulated ACTH secretion caused by an ACTH-secreting pituitary adenoma (1). The significant associated morbidity, such as increased tissue fragility, poor wound healing, hypertension and diabetes mellitus, demands a proper medical intervention (2). Trans-sphenoidal surgery is currently the first line of treatment, while secondary options consist of irradiation therapy either alone or in combination with adrenolytic agents (3-

6). Unfortunately, none of the current treatment modalities ensure a full and permanent cure, as evidenced by the number of patients developing recurrent Cushing's disease (7). The absence of an effective medical treatment has prompted physicians to explore new medical strategies, preferably based on fundamental and (patho-) physiological pathways, in the hope of increasing the curation chances in this group of patients. The physiological role of somatostatin (SRIF) in the regulation of anterior pituitary function (8-11), its equivocal effects on ACTH release (12, 13), and the current use of SRIF-analogs in patients with anterior pituitary tumours (14), has led to the exploration of SRIF-analogs in patients with (recurrent) Cushing's disease. To date, five G-protein coupled SRIF-receptors have been cloned (sst_1-sst_5) and six gene products are currently known (10, 15). The receptor subtypes sst_{1-5} produce single gene products, while sst_{2A} (long form) and sst_{2B} (short form) originate from a common precursor mRNA which is spliced at the carboxy terminus (16). While *in vitro* data demonstrate the presence of sst expression in corticotroph adenomas, the sst_2-preferential analog octreotide (OCT) appears to inhibit ACTH release in Nelson's syndrome and in some patients harboring ectopic ACTH-producing tumours, but rarely in patients with Cushing's disease (17, 18). These observations are in agreement with the observation that almost all ACTH-secreting pituitary adenomas, *i.e.* patients with untreated Cushing's disease, cannot be visualized by SRIF-receptor (sst) scintigraphy using ^{111}In-diethylenetriamine pentaacetic acid (DTPA) octreotide (19, 20), whereas ^{111}In-DTPA scintigraphy is positive in patients with Nelson's syndrome (19, 21). Apparently, ACTH release from corticotrophinomas is only sensitive to OCT in the absence of peripheral feedback regulation by glucocorticoids, suggesting that the sst_2 might be down-regulated when cortisol levels are high. Additional *in vitro* evidence for this hypothesis comes from studies using primary cultures of human corticotroph adenomas, in which glucocorticoids down-regulated the response of corticotrophin releasing hormone (CRH)-induced ACTH secretion to OCT (22).

To explore the possible role of novel SRIF-analogs in the medical treatment of Cushing's disease, we have further evaluated the potential signif-

icance of sst subtypes expressed in human corticotroph adenomas and determined the effects of the novel multiligand SRIF-analog, SOM230, on ACTH release in primary cultures of human corticotroph adenomas (23). SOM230, compared with OCT, has a 30-, 5- and 40-times higher affinity to sst_1, sst_3 and sst_5 receptors, respectively, and 2.5 times lower affinity to sst_2 (24). Its elimination half-life of 23 hours makes this compound suitable for clinical application (25, 26). On the basis of the observed selective expression of the sst_5 receptor in this series of human corticotroph adenomas, the very high affinity of SOM230 for sst_5 receptors, and the inhibition by SOM230 of basal ACTH release by human corticotroph adenoma cells, even when the cells were pretreated with DEX, it was hypothesized that this multiligand SRIF-analog may become a new medical treatment modality in a subgroup of patients with pituitary dependent Cushing's disease.

The present study was carried out to further evaluate the role of sst_5 in suppressing ACTH secretion from pituitary corticotrophs, with particular emphasis on the role of glucocorticoids in regulating sst_2 and/or sst_5-mediated ACTH suppression. Studies were carried out by using the mouse ACTH-producing AtT-20 corticotroph tumour cell line, which is known to express mainly sst_{2A+2B} and sst_5 subtypes (16, 27, 28).

Methods

Cell culture

AtT-20/D16V mouse tumour cells (Dr. J. Tooze; European Organization Molecular Biology) were routinely passaged by trypsinization as described in detail previously (29). The cells were maintained in 75 cm^2 flasks in Dulbecco's Minimal Essential Medium (DMEM), supplemented with non essential amino acids, sodium pyruvate (1 mmol/L), 10% fetal calf serum (FCS), penicillin ($1x10^5$ U/L), fungizone (0.5 mg/L), L-glutamine (2 mmol/L), and sodium bicarbonate (2.2 g/L), pH 7.6. The cells were cultured at 37° C in a CO_2-incubator. Media and supplements were obtained from GIBCO Bio-cult Europe (Invitrogen, Breda, The Netherlands). For crude cell membrane preparations, AtT-20 cells were scraped

and centrifuged at 4°C for 5 min at 1000g. The cell pellet was either stored at -80°C or used directly. For ACTH release studies, the cells were seeded at a density of 20,000 cells per well in 1mL of culture medium. After 72 h, the medium was changed and a 2 h incubation without or with the SRIF-analogs in the presence of CRH (10 nM) was performed. In order to evaluate the effect of glucocorticoids or SRIF-analogs on SRIF-analog [SRIF-14, OCT, SOM230 and BIM-23268 (Table 1)] induced inhibition of ACTH release, the cells were pre-treated for 48 h with the synthetic glucocorticoid DEX (10 nM) or 72 h with SRIF-14, OCT or SOM230 (all 10 nM), respectively. After this preincubation, the medium was changed and the effect of SRIF-analogs on CRH-induced ACTH secretion was evaluated as described above. At the end of the incubations the medium was collected and stored at -20° C until hormone determination.

Table 1. Binding selectivity of SRIF-analogs for the five human sst.

Compound	Binding affinity (IC_{50}, nM)				
	sst_1	sst_2	sst_3	sst_4	sst_5
Somatostatin-14	2.3	0.2	1.4	1.8	1.4
Octreotide	280	0.4	7.1	>1000	6.3
SOM230	9.3	1.0	1.5	>100	0.2
BIM-23268	18	15	62	16	0.4

Data are from radioligand binding assays to membranes from transfected CHO-K1 cells (25, 61) and African green monkey kidney cells (25) expressing the different human sst subtypes. Values are from IPSEN (Culler, M.D.), Shimon (61) and from Bruns et al. (25).

Radioligand binding assay

As previously described (28), the cells were resuspended in binding assay buffer (0.5% (w/v) bovine serum albumin (BSA), 10 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] (HEPES), pH 7.5) by homogenisation with a Polytron homogeniser (Kinematica AG) at 50 Hz for 30 s. 150 µl of cell homogenate (ca. 10000-25000 cells) was incubated with 50 µl of [^{125}I]LTT-SRIF-28 (2175 Ci/mmol, 25-75 pM final concentration) in binding assay buffer containing $MgCl_2$ (5 mM) and bacitracin (20 µg/ml), and either 50 µl binding assay buffer alone (total binding), supplemented with 1 µM SRIF-14 (non specific binding) or with increasing concentrations of SOM230 or OCT. Experiments were conducted in triplicate. Incubation was terminated after 1 h at room temperature by vacuum filtration through glass fibre filters pre-soaked in 0.25% (w/v) polyethyleneimine. The filters were washed 3 times with ice-cold 10 mM Tris-HCl buffer containing 154 mM NaCl (pH 7.4) and dried. Bound radioactivity was measured in a Packard TopCount using liquid scintillation (65% counting efficiency).

[^{35}S]GTPγS binding assay

As previously described (30), the cell pellet was re-suspended in 10 mM HEPES, pH 7.5, by Polytron homogenisation at 50 Hz for 30 s, and centrifuged at 4°C for 30 min at 15000*g*. The microsome pellets were re-suspended in assay buffer (10 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 10 µg/ml bacitracin, pH 7.4), and either stored at -80°C or directly used. 100 µl per well of the microsome preparation (ca. 75000 cells) were incubated in 96-well plates with [^{35}S]GTPγS (1030 Ci/mmol, 100-200 pM final concentration) in assay buffer containing 1 µM GDP and triplicates of either: assay buffer (basal), 10 µM GTPγS, SOM230 or OCT at increasing concentrations. After 5 min preincubation, 1.5 mg per well of wheatgerm agglutinin (WGA) scintillation proximity assay (SPA) beads (Amersham) were added (beads in 50 mM Tris-HCl, 0.1% (w/v) sodium azide, pH 7.4), the plates sealed, incubated for 30 min at room temperature, and centrifuged for 10 min

at 1000*g*. During assay incubation cell membranes bind to WGA, effectively immobilizing the receptor-bearing membranes on to the SPA bead. The binding of [^{35}S]GTPγS to such immobilized receptors brings the isotope into close proximity with the scintillant, which is incorporated within the bead. This allows the emitted radiation to stimulate the scintillant to emit light, which was measured (cpm) in a Packard TopCount. Percent stimulation of specific basal [^{35}S]GTPγS binding was calculated as: 100 x [(experimental - basal)/(basal - non-specific)].

Quantitative PCR

Quantitative PCR was performed as previously described (31). Briefly, poly A^+ mRNA was isolated during Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway) from AtT20/D16V cell pellets containing $0.5x10^6$ cells per sample. cDNA was synthesized using the poly A^+ mRNA, which was eluted from the beads in 40 µl H_2O for 2 minutes at 65° C, using Oligo $(dT)_{12-18}$ Primer (Life Technologies). One-twentieth of the cDNA library was used for quantification of the sst subtype mRNA levels. A quantitative RT-PCR was performed by TaqMan® Gold nuclease assay (The Perlin-Elmer Corporation, Foster City, CA) and the ABI PRISM® 7700 Sequence Detection System (The Perkin-Elmer) for real-time amplification, according to the manufacturer's instructions. The assay was performed using 15 µl TaqMan® Universal PCR Master Mix (Applied Biosystems, the Netherlands), 300 nM forward primer, 300 nM reverse primer, 200 nM probe and 10 µl cDNA template, in a total reaction volume of 25 µl. The detection of pro-opiomelanocortin (POMC) mRNA was performed as a control for the negative feedback regulation by glucocorticoids on POMC gene expression. The detection of hypoxanthine phosphoribosyl transferase (HPRT) mRNA served as a control and was used for normalisation of the POMC and sst subtype mRNA levels. The specific mouse primer sequences (Biosource, Nivelles, Belgium) that were used included:
HPRT forward 5'-TGAAGAGCTACTGTAATGATCAGTCAAC-3'
HPRT reverse 5'-AGCAAGCTTGCAACCTTAACCA-3'
POMC forward 5'-ACCTCACCACGGAGAGCAAC-3'

POMC reverse 5'-GCGAGAGGTCGAGTTTGCA-3'
Sst$_{2A}$ forward 5'-TGAGTGGTACGGAGGATGGG-3'
Sst$_{2A}$ reverse 5'-CTCCGTGGTCTCATTCAGCC-3'
Sst$_{2B}$ forward 5'-CAAGGCAGACAATTCACAATCC-3'
Sst$_{2B}$ reverse 5'-GTTTCTGCCGGGCAGCT-3'
Sst$_5$ forward 5'-GCGCTCAGAACGCAAGGT-3'
Sst$_5$ reverse 5'-CAGCAGCCCACGAACACC-3'

The probe sequences that were used included:
HPRT 5'-FAM-TGCTTTCCCTGGTTAAGCAGTACAGCCC-TAMRA-3'
POMC 5'-FAM-TGCTGGCTTGCATCCGGGC-TAMRA-3'
Sst$_{2A}$ 5'-FAM-AGAGGAGCGACAGTAAGCAGGACAAATCC-TAMRA-3'
Sst$_{2B}$ 5'-FAM-ATTGCCTGGGTGTGACCTGGTGGA-TAMRA-3'
Sst$_5$ 5'-FAM-ACTCGCATGGTGGTGGTAGTGGTGC-TAMRA-3'

The relative amount of POMC, sst$_2$ and sst$_5$ mRNA was determined by means of a standard curve generated in each experiment from known amounts of mouse genomic DNA. For the determination of the amount of HPRT mRNA, a standard curve was obtained by including dilutions of a pool cDNAs known to contain HPRT. The amount of POMC, sst$_2$ and sst$_5$ mRNA was calculated relative to the amount of HPRT and is given in arbitrary units.

Sst membrane binding studies

The method of membrane isolation and the reaction conditions were previously described (32). Briefly, membrane preparations (corresponding to 30-50 μg protein) of cultured AtT-20 cells, in the presence or absence of 10nM DEX (48 h), were incubated in a total volume of 100 μl at room temperature for 60 min with increasing concentrations of [^{125}I-Tyr^{11}]-SRIF-14 or [^{125}I-Tyr^3]-Octreotide with and without excess (1 μM) of unlabeled SRIF-14 or Octreotide, respectively, in Hepes buffer (10 mM Hepes, 5 mM $MgCl_2$ and 0.02 g/L bacitracin, pH 7.6) containing 0.2% BSA. After the incubation, 1 mL ice-cold Hepes buffer was added to the reaction mixture, and membrane-bound radioactivity was separated from unbound by

centrifugation during 2 min at 14,000 rpm in a Eppendorf microcentrifuge. The remaining pellet was washed twice in ice-cold Hepes buffer, and the final pellet was counted in a γ-counter. Specific binding was taken to be total binding minus binding in the presence of 1 μM unlabeled SRIF-14 or Octreotide. As a control for binding, rat brain cortex membranes were used.

Hormone determination

Mouse immunoreactive (ir-) ACTH concentrations were determined by a non-isotopic, automatic chemiluminescence immunoassay system (Immulite, DPC Inc.), as described previously for the detection of rodent ir-ACTH (33). The intra- and inter-assay coefficients of variation (CV) for ACTH were 5.6% and 7.8%, respectively. Under the conditions employed, the assay detects 1 fmol/tube of ir-ACTH. Dilution curves of the samples were parallel with those of the standard, and in addition, biological specificity of the results was in agreement with hypothalamic-pituitary-adrenal physiology.

Test-substances

Octreotide (OCT, Sandostatin®) was obtained from Novartis Pharma A.G., (Basel, Switzerland). SOM230 was synthesized at Novartis Pharma A.G. Somatostatin-14 was purchased from Sigma Chemical Co. (St. Louis, MO). BIM-23268, an sst_5-subtype specific analog, was synthesized at IPSEN (Massachusetts, USA). Dexamethasone was derived from the pharmacy department of the Erasmus MC (Rotterdam, the Netherlands). Corticotropin-releasing hormone (CRH) was purchased from Ferring (Hoofddorp, the Netherlands). [^{125}I]LTT-SRIF-28 was custom synthesised by ANAWA (Wangen, Switzerland). [^{125}I-Tyr^{11}]-SRIF-14 was purchased from Amersham (Houten, The Netherlands). The SRIF-analog [Tyr^3]-Octreotide was iodinated with ^{125}I by the chloramine-T method and purified by HPLC, as described previously in detail (34). Specific radioactivity of all radioligands yielded approximately 2000 Ci/mmol. [^{35}S]GTPγS was purchased from Amersham (Freiburg, Germany).

Statistical analysis

The statistical significance of the difference between mean values regarding the effects of the SRIF-analogs on ACTH release was determined by using one-way analysis of variance (ANOVA). When significant overall effects were obtained by this method, comparisons were made using Newman-Keuls multiple comparisons test. The unpaired Student t-test was chosen to analyze for statistical significance in the experiments determining the effects of DEX treatment on POMC and sst mRNA expression levels. pKd, EC_{50} and IC_{50} values were determined by non-linear regression curve analysis of the concentration-effect responses using the computer programs ActivityBase and GraphPad Prism. The unpaired Student t-test was chosen to analyze differences in concentration-effect curves. Data are reported as means ± SEM of the indicated n values, unless otherwise specified.

Results

Radioligand and [^{35}S]GTPγS binding assays

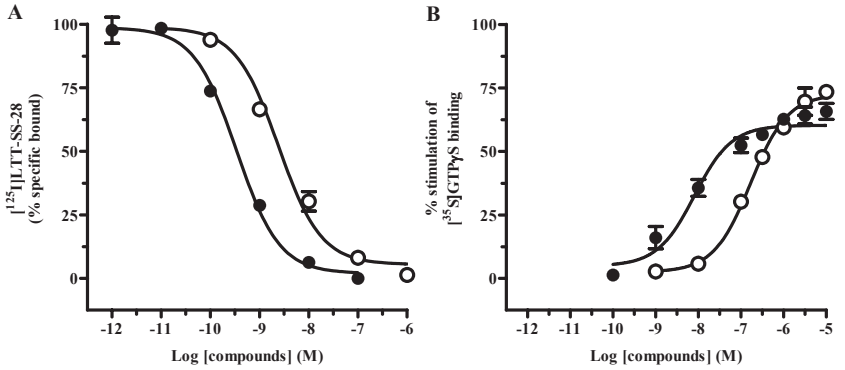

Figure 1. Inhibition of radioligand binding in AtT-20 cell membranes (A). Crude membrane preparations were incubated with [^{125}I]LTT-SRIF-28, and increasing concentrations of SOM230 (●) and OCT (○). Data are expressed as percentage of specific binding. The data points represent the mean ± SEM (bars) of data from 3-8 different experiments run in triplicate. (B). Stimulation of [^{35}S]GTPγS binding in AtT-20 cell membranes. Dose-Response effects of SOM230 (●) and OCT (○). Data are expressed as percentage stimulation of specific basal [^{35}S]GTPγS binding. The data points represent the mean ± SEM (bars) of data from 3-12 different experiments run in triplicate

The binding properties of SOM230 and OCT at [^{125}I] Leu^8 Trp^{22} Tyr^{25} SRIF-28 (LTT-SRIF-28) labeled sites in AtT-20 cell membranes were es-

tablished in competition experiments. As shown in figure 1A, SOM230 had a 7-fold higher ligand binding affinity than OCT for [^{125}I]LTT-SRIF-28 labeled sites in AtT-20 cell membranes [IC_{50} 0.18 nM (pKd 9.74 ± 0.08, n=8) vs. 1.2 nM (pKd 8.92 ± 0.03, n=3), respectively; $p<0.0001$]. In microsome preparations from AtT-20 cells, both SOM230 and OCT produced a concentration-dependent increase in [^{35}S]GTPγS binding (Fig. 1B). Interestingly, SOM230 showed a clear 19-fold higher potency in stimulating GTPγS binding as compared with OCT [EC_{50} 8.71 nM (pKd 8.09 ± 0.11, n=12) and 169.8 nM (pKd 6.78 ± 0.07, n=3), respectively; $p<0.0001$].

Effect of SRIF-14 and SRIF-analogs on CRH-induced ACTH secretion in AtT-20 corticotroph cells, with or without glucocorticoid pre-treatment.

Full dose-response curves were performed to ascertain which concentration of DEX and CRH should be used in our experiments. As can be seen in Figure 2, DEX concentration-dependently suppressed POMC mRNA expression in AtT-20 cells, after 24 h (Fig. 2A) and 48 h (Fig. 2B), whereby 10nM was most effective. CRH induced ACTH release by AtT-20 cells in a dose-dependent way as well, which reached its maximum effect at 10 nM CRH (Fig. 2C). Because of the physiological negative feedback loop within the hypothalamus-pituitary-adrenal axis, the effect of 48 h DEX (10 nM) pre-treatment was assessed (Fig. 2D). ACTH release by AtT-20 cells was decreased when cultured for 48 h with DEX (from 1213 ± 32 to 75 ± 1.8 fmol/L*well; $p<0.0001$). Nevertheless, DEX-treated AtT-20 cells remained responsive to CRH (10 nM) stimulation (Fig. 2D). In order to evaluate the effects of DEX pre-treatment on CRH-induced ACTH release in AtT-20 cells, we first investigated the inhibitory potencies of the SRIF-analogs on CRH-induced ACTH-release in AtT-20 cells, under basal conditions. Each SRIF-analog (100 nM) potently inhibited CRH-induced ATCH release varying between 58% and 75% suppression (BIM-23268, $p<0.05$ vs OCT and SRIF-14; SOM230 vs OCT, $p<0.05$). The dose-dependent inhibition of ACTH release by SRIF-14 (Fig. 3A) and SOM230 (Fig. 3B) was not affected by 48h DEX (10nM) and the corresponding IC_{50} values for SRIF-14 (1.3 nM without vs. 0.7 nM with DEX;

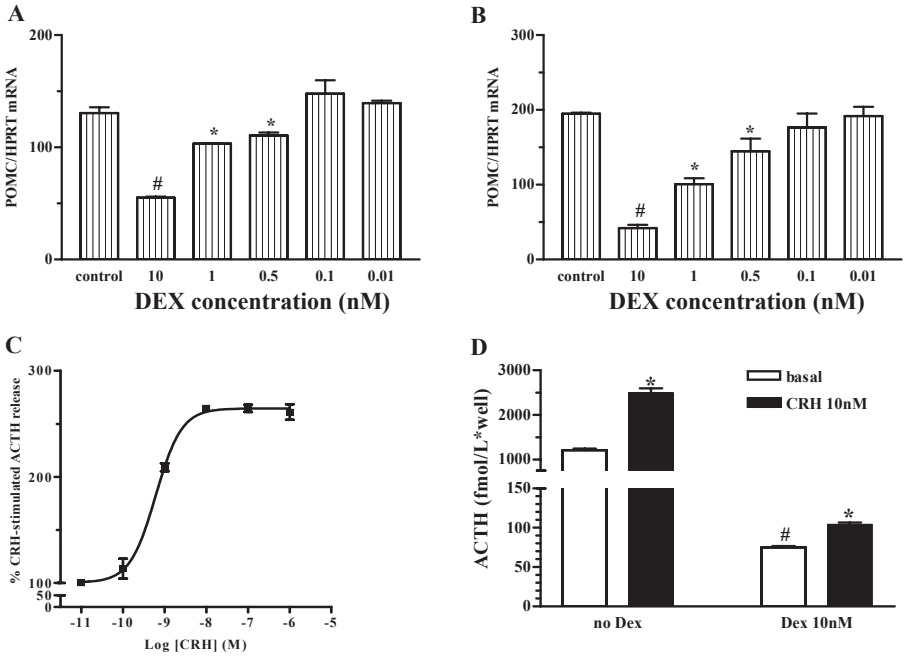

Figure 2. Effect of glucocorticoids and CRH on POMC mRNA and ACTH release in AtT-20 corticotroph tumour cells. Cells were pre-incubated during 24 (A) and 48h (B) with dexamethasone (range 0.01-10 nM DEX). Subsequently, Poly A+-mRNA was isolated, which was used to determine the expression levels of POMC by quantitative PCR (corrected for HPRT). Values represent percent change of POMC mRNA transcripts under DEX treatment compared with control, and are the mean ± SEM of two independent experiments. #, $p<0.001$ vs control, 0.1 and 0.01nM DEX; *, $p<0.05$ vs control, 0.1 and 0.01nM DEX. C) Concentration effect curve of 3 h incubation with CRH (0.01-1000 nM) on ACTH release in AtT-20 cells. Values represent percent change of ACTH release compared with control, and are the mean ± SEM of two independent experiments run in quadruplicate. D) Effect of Dexamethasone (10 nM; 48 h) on CRH-induced ACH release in AtT-20 cells. After 48 h, the medium was refreshed and the cells were incubated for 2 h in the absence or presence of DEX and CRH (10nM) after which the medium was collected for ACTH determination. Values are expressed as mean ± SEM of four wells per treatment group. The left part depicts CRH induced ACTH release in the absence of DEX (no Dex); the right part of the graph depicts CRH-induced ACTH release after 48h 10nM DEX-treatment. #, $p<0.0001$ compared with basal ACTH release in the absence of DEX; *, $p<0.001$ CRH vs basal.

p=ns) and SOM230 (0.06 nM without vs. 0.07 nM with DEX; p=ns) remained unchanged. Interestingly, the concentration-response curve for the inhibitory effects of BIM-23268 on CRH-induced ACTH release showed a shift to the left, indicating increased potency, when pre-treated with DEX, with the IC_{50} value shifting 34-fold from 3.4 to 0.1nM in the pres-

ence of DEX ($p<0.05$; Fig. 3C). In contrast, DEX pre-treatment caused the concentration-response curve of OCT to shift to the right, indicating decreased potency. The IC_{50} indicates a 20-fold shift from 0.2 nM without DEX to 4.3 nM in the presence of DEX ($p<0.05$; Fig. 3D). Both 100 nM

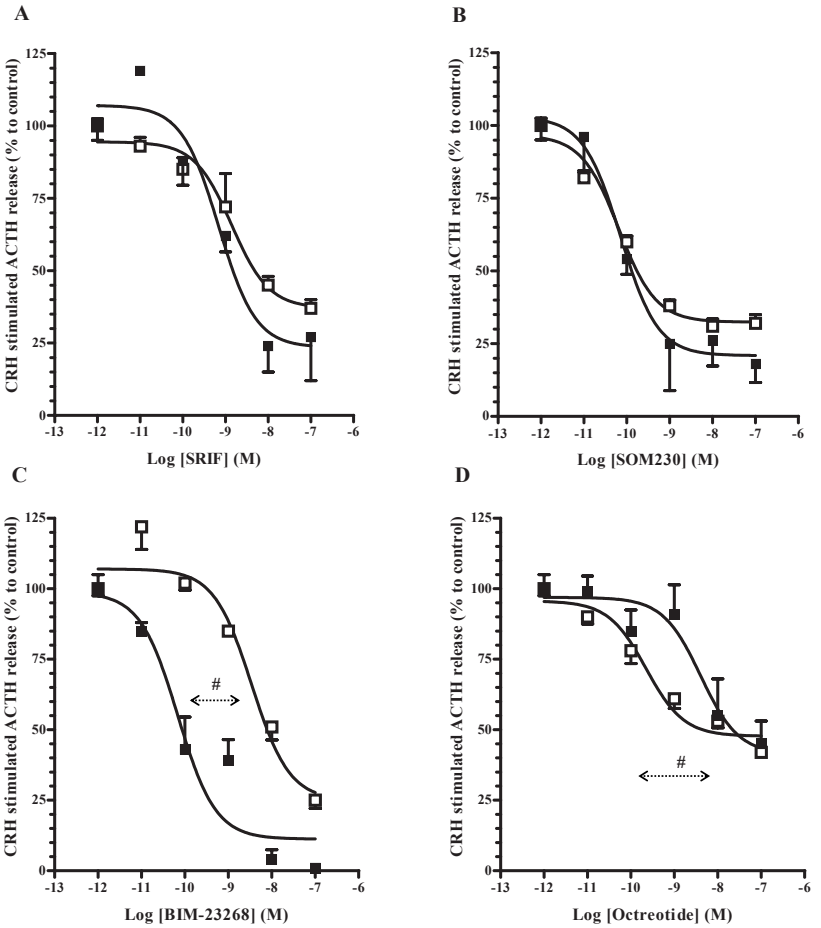

Figure 3. Effect of glucocorticoids on concentration-dependent inhibition of CRH-induced ACTH release in mouse corticotroph tumour cells by different SRIF-analogs. AtT-20 cells were pre-incubated during 48 h with 10 nM DEX. After 48 h, medium was collected and exchanged for new medium containing 10 nM CRH and different concentrations of SRIF (A), SOM230 (B), BIM-23268 (C) or OCT (D). Incubation was performed for an additional 2 h and the medium was collected for ACTH determination. Calculation of the IC_{50} values was performed using the GraphPad Prism (San Diego, CA) computerized program and the unpaired Student t-test was chosen to analyze differences in IC_{50} values. Control □, DEX 10nM ■. #, $p<0.05$.

BIM-23268 and 100 nM SOM230 showed significantly enhanced sup-

pressive effects on CRH-induced ACTH release in the presence of DEX ($p<0.01$ and $p<0.05$ vs without DEX, respectively). No differences in the maximal inhibitory effects were observed when the cells were incubated with 100 nM OCT without or with DEX; however, as shown in figure 4, the inhibitory effect of 1 nM OCT on CRH-induced ACTH release was almost completely abolished by DEX treatment (-39 ± 2% without vs. -9 ± 6% with DEX; $p<0.05$). In contrast, DEX treatment enhanced the ability of 1 nM BIM-23268 (Fig. 4: -15 ± 1% without vs. -60 ± 7% with DEX; $p<0.01$) to suppress CRH-induced ACTH release. DEX did not affect the inhibitory effects of 1 nM SOM230. Both SOM230 and BIM-23268 were significantly more efficacious than OCT in suppressing ACTH release under DEX treatment, not only at supra physiological but also at physiological concentrations ($p<0.05$, BIM-23268 and SOM230 vs OCT).

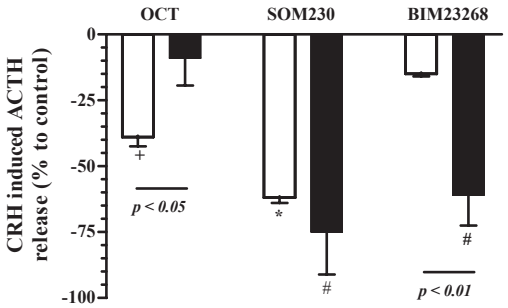

Figure 4. Effect of glucocorticoids on SRIF and SRIF-analog mediated inhibition of CRH-induced ACTH release by AtT-20 cells. Cells were pre-incubated with or without 10nM DEX. After 48 h, the medium was refreshed and the cells were incubated for 2 h in the absence or presence of 10nM DEX, CRH (10nM) and the indicated compounds at a concentration of 1nM. Subsequently, medium was collected for ACTH determination. Values are expressed as mean ± SEM of four wells per treatment group. Control □, DEX 10nM ■. # , $p<0.05$ SOM230 and BIM-23268 vs OCT. +, $p<0.05$ OCT vs BIM-23268. *, $p<0.05$, SOM230 vs OCT and BIM23268.

Effect of glucocorticoid treatment on sst$_2$ + sst$_5$ mRNA levels

To determine whether glucocorticoids display a regulatory role on both sst$_2$ isoforms and/or sst$_5$ mRNA expression levels, AtT-20 cells were exposed to 10 nM DEX for 24 and 48 h. As shown in figure 5, DEX significantly suppressed POMC mRNA levels after 24 and 48 h (-55 ± 2% and -74 ± 2%, respectively: both $p<0.001$ to control). Sst$_{2A}$ and sst$_{2B}$ mRNA

levels were potently suppressed after 24 h of DEX treatment by 30 ± 6% and 45 ± 4%, respectively (Figure 5, left panel: $p<0.001$), which remained significantly lower after 48 h (Figure 5, right panel). In contrast, sst_5 mRNA expression remained constant after 24 h of DEX and was not significantly affected (20 ± 8%; p = ns) after 48 h DEX treatment.

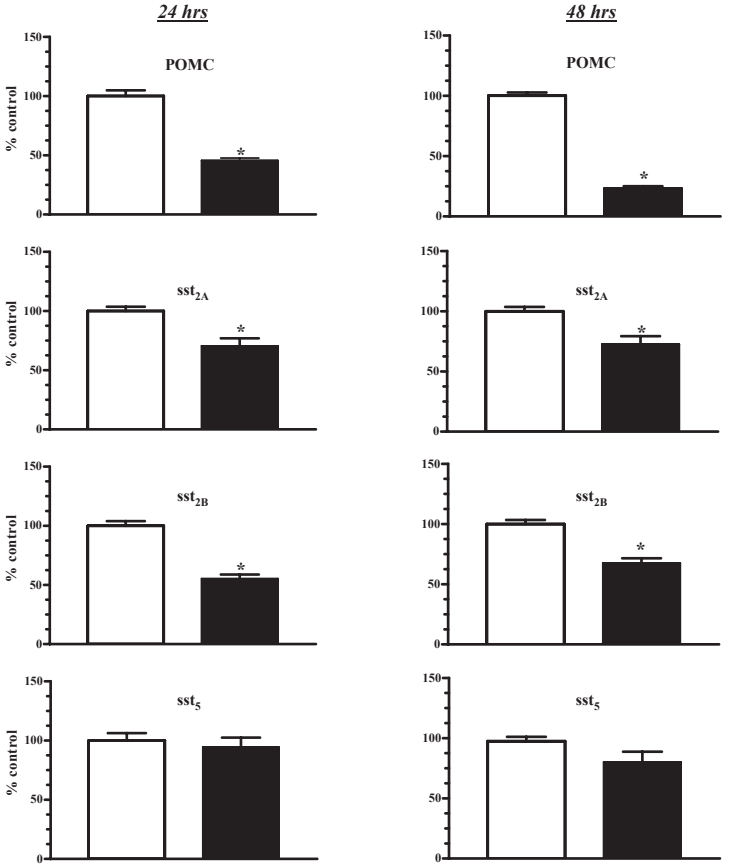

Figure 5. Effect of glucocorticoids on sst mRNA expression levels in AtT-20 pituitary adenoma cells. AtT-20 cells were pre-incubated for 24 h (left panel) and 48 h (right panel) without or with 10nM DEX. Poly A+-mRNA was isolated from untreated and treated AtT-20 cells, which was used to determine the expression levels of POMC or sst mRNA by quantitative PCR (corrected for HPRT). Values represent percent change of the different mRNA transcripts under DEX treatment compared with control, and are the mean ± SEM of three independent experiments. Control □, DEX 10nM ■. *, $p<0.001$.

Effect of glucocorticoid treatment on sst binding sites

To determine whether glucocorticoids display a regulatory role on sst_2

and/or sst$_5$ at the protein level as well, AtT-20 cells were cultured in the presence or absence of 10nM DEX for 48 h and subsequently collected for cell membrane binding assays with two different radiolabeled somatostatin analogs: [^{125}I-Tyr^3]-Octreotide, displaying superior binding affinity for only sst$_2$, and [^{125}I-Tyr^{11}]-SRIF-14, which can bind to both sst$_2$ and sst$_5$ with good affinity. Maximum binding capacity (Bmax) was dramatically suppressed by 72% in the presence of 10nM DEX when [^{125}I-Tyr^3]-Octreotide was used as radioligand (Fig. 6A; Bmax 1404 to 390 fmol/mg in the absence and presence of DEX, respectively). As depicted in Figure 6B, DEX attenuated radioligand binding only by 17% when [^{125}I-Tyr^{11}]-SRIF-14 was used as radioligand (Bmax 1581 and 1315 fmol/mg in the absence and presence of 10nM DEX, respectively).

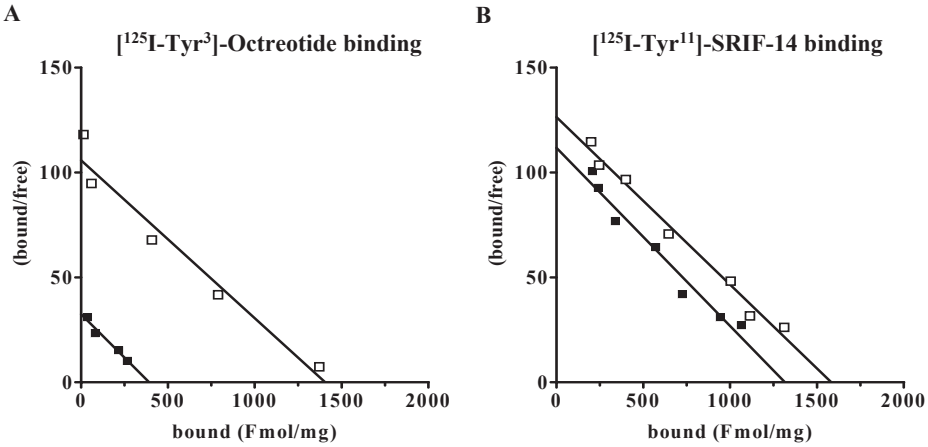

Figure 6. Sst membrane binding studies. A) Scatchard analysis of [^{125}I-Tyr^3]-Octreotide binding to cell membranes of AtT-20 cells, cultured in the absence (Kd= 0.1 nM, n= 1404 fmol/mg) or presence of 10 nM DEX (48 h; Kd= 0.7 nM, n= 390 fmol/mg). B) Scatchard analysis [^{125}I-Tyr^{11}]-SRIF-14 binding to cell membranes of AtT-20 cells, cultured in the absence (Kd= 0.1, n= 1581 fmol/mg) or presence of 10 nM DEX (48 h; Kd= 0.1 nM, n= 1315 fmol/mg). Control □, DEX 10nM ■.

Effect of SRIF-analog pre-treatment

First, it should be noted that SRIF-analog pre-treatment lowered the inhibitory effects of all SRIF-analogs (10 nM) on CRH-induced ACTH release (from 47-69% to 18-54% range of suppression). In order to evaluate the effect of continued exposure of AtT-20 cells to a maximal inhibitory concentration of the different SRIF-analogs, AtT-20 cells

were preincubated for 72 h with either SRIF-14, OCT or SOM230 (10 nM), followed by 2 h incubation with CRH (10 nM) and either SRIF-14, OCT, SOM230 and/or BIM-23268 (all 10 nM). As depicted in Figure 7A, the inhibitory effects of SOM230 (-48%) and BIM-23268

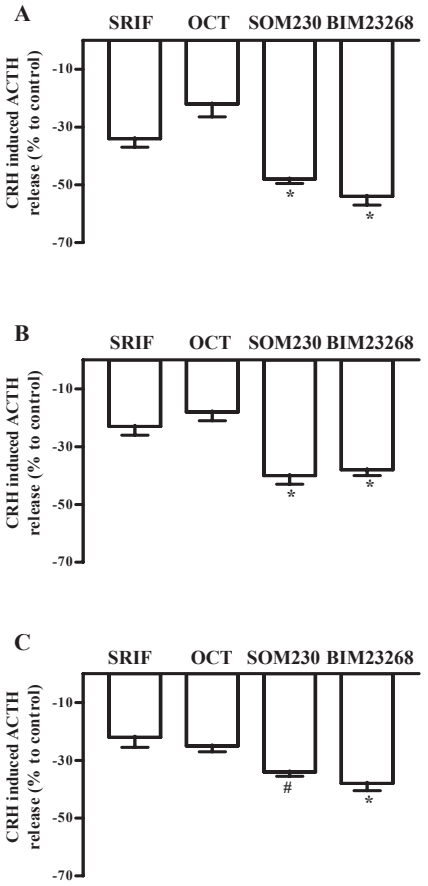

Figure 7. Effect of prior SRIF-analog treatment on the SRIF-analog mediated inhibition of CRH-induced ACTH release by AtT-20 cells. Cells were pre-incubated with 10nM SRIF (A), OCT (B) or SOM230 (C). After 72 h, the medium was refreshed and the cells were incubated for 2 h in the absence or presence of 10nM SRIF-analog, CRH (10nM) and the indicated compounds (10nM), after which the medium was collected for ACTH determination. Values are expressed as mean ± SEM of four wells per treatment group. *, $p<0.01$ vs OCT and SRIF; #,$p<0.05$ vs OCT and SRIF.

(-54%) appeared to be two fold more efficacious as compared with OCT (-22%; *$p<0.01$ vs SOM230 and OCT) when AtT-20 cells were treated for 72h with SRIF-14. Comparable superior suppressive ef-

fects by SOM230 and BIM-23268, compared with OCT, were observed when cells were pre-treated with OCT (Fig. 7B) or SOM230 (Fig. 7C).

Discussion

Because purified populations of corticotrophs are difficult to prepare and require substantive amounts of pituitary tissue, we based our study on the mouse pituitary corticotroph (AtT-20/D16V) cell line after first confirming by quantitative RT-PCR the expression of sst_{2A}, sst_{2B} and sst_5 mRNA. Although it is appreciated that cell lines are not necessarily representative of their parent cell types, we reasoned that this clonal population, which has been studied intensively as a well-accepted cellular model for corticotrophs (35), would provide a useful model in which to explore fundamental mechanisms of the effects of SRIF (-analogs) on ACTH release and the role of sst subtypes herein. In the present study predominance of sst_2 and sst_5 was found as both OCT and SOM230 showed high affinity binding. These observations are well in agreement with recent extensive pharmacological studies that indicated sst_2 and sst_5 are mainly expressed with no or negligible presence of sst_1, sst_3 and sst_4 in AtT-20 cells (28). The inhibitory effects of SRIF-14, SOM230, OCT and BIM-23268 on CRH-induced ACTH release in AtT-20 cells, are well in agreement with the concept that both sst_2 and sst_5-binding SRIF-analogs potently inhibit CRH-induced ACTH release by AtT-20 cells (36). Moreover, the maximal inhibitory effect of the two SRIF-analogs that bind with very high affinity to sst_5, *i.e.* SOM230 and BIM-23268, was significantly higher as compared with OCT. This apparent functional superiority of sst_5 over sst_2 seems to confirm recent observations by Cervia and coworkers (30), as well as our membrane and GTPγS binding results showing the superior profile of SOM230 compared to OCT. In the present study we found that glucocorticoid treatment induced remarkable differences with respect to the role of sst_2 and sst_5 in regulating ACTH release. We observed a profound difference in efficacy between the sst_2 specific analog, OCT, and the multiligand SRIF-analog, SOM230, and the sst_5 preferring analog, BIM-23268. Since both

SOM230 and BIM-23268 still potently inhibited CRH-induced ACTH release under DEX treatment whereas the suppressive effects of OCT were almost completely blocked in the "physiological" nanomolar range, it is suggested that sst_2 is down-regulated by glucocorticoids treatment while sst_5 is more resistant. The intriguing differences in the functional properties of sst_2 and sst_5 in mediating ACTH release under DEX pressure is further supported by our observations that both the IC_{50} values for OCT and BIM-23268 during DEX treatment shift toward their sst_5 binding affinity, *i.e.* a 20-fold decrease and 34-fold increase in their potency to suppress ACTH release, respectively. Our data not only confirm earlier observations that glucocorticoid treatment abolishes the inhibitory effect of OCT on ACTH release *in vitro* (22) but also seem well in agreement with other reports which demonstrate that SRIF only suppressed ACTH release by rat pituitary cells from long-term adrenalectomized rats (37) or when the cells were cultured in the absence of glucocorticoids (11, 38), One group did observe ACTH suppression by SRIF-28, which is known to have preferable binding affinity for sst_5, in a primary cell culture from a Nelson's tumour and from an ACTH-secreting pituitary adenoma (39, 40). However, in patients with Nelson's syndrome and adrenal insufficiency of different origin, both SRIF and OCT lower ACTH secretion (14, 41-43), again suggesting that glucocorticoids influence the sensitivity of the corticotroph cells for SRIF and OCT. Because glucocorticoids are also well known to mediate biologic effects through regulation of gene expression, it was of interest to test the effects of glucocorticoids on sst_2 and sst_5 gene expression in AtT-20 cells. The primary transcript of the sst_2 gene is alternatively spliced in a long (sst_{2A}) and a short (sst_{2B}) form (16, 27, 44-46). The current mRNA data again support the concept that glucocorticoid treatment differentially influences sst_{2A+2B} expression as compared with sst_5. It is known that the mouse sst_2 gene promoter sequence is the only sst receptor that has been shown directly to be transcriptionally regulated by glucocorticoids (47, 48), whereas the mouse sst_5 gene possesses multiple glucocorticoid responsive elements half-sites (49). Therefore, the immediate and powerful suppression of both sst_{2A+2B} suggests a direct effect of DEX at the transcriptional level,

whereas sst_5 could be regulated in a different way. Support for transcriptional down-regulation of sst subtype$_{1+2+3}$ mRNA expression by glucocorticoids comes from studies in rat pituitary GH_4C_1 cells (50). Influencing mRNA stability, could be involved as well since the addition of a RNA synthesis inhibitor produces no disruption of the ability of DEX to suppress either sst_2 or sst_3 mRNA levels in cultured rat pituitary cells (51). Our observation that sst_2 and sst_5 receptors might be regulated differently by glucocorticoids was also recently observed by Park and coworkers: in a rat model, DEX inhibited sst_2 mRNA expression both *in vivo* and *in vitro*, but enhanced sst_5 mRNA expression (51). Moreover, earlier observations already demonstrated that glucocorticoids lower [^{125}I-Tyr^{11}]-SRIF-14 binding in AtT-20 cells, but sst receptors were not characterized (52). Our data confirm that [^{125}I-Tyr^{11}]-SRIF-14 binding is attenuated in AtT-20 cells after DEX treatment. However, [^{125}I-Tyr^{3}]-Octreotide binding, *i.e.* the presence of only sst_2 receptor subtypes, was reduced by almost 75% by DEX, indicating that sst_2 at the protein level is dramatically decreased. Since [^{125}I-Tyr^{11}]-SRIF-14 binding displays both sst_2 and sst_5 binding affinity and is only slightly lowered by DEX, it becomes suggestive that this universal radioligand represents predominantly sst_5 receptors when AtT-20 cells are treated by glucocorticoids. These experiments clearly support our mRNA data and demonstrate at the protein level that sst_5, compared with sst_2, seems less sensitive to DEX treatment as well. The down regulation of sst_{2A+2B} mRNA and protein levels in AtT-20 cells by glucocorticoids, may be an explanation for the lack of efficacy of OCT in lowering ACTH and cortisol levels in patients with untreated Cushing's disease (18). Thus, the observed ability of sst_5 to suppress ACTH release in AtT-20 cells, which appears to be relatively resistant to glucocorticoids, might be a new target for therapeutical agents that could lower ACTH and cortisol levels in a subgroup of patients with Cushing's disease. Furthermore, we propose that sst_2 + sst_5 preferring SRIF-analogs, such as SOM230, might become of therapeutic interest in Cushing's disease as well. The suppression of ACTH levels by activation of sst_5 in patients with Cushing's disease might lower cortisol levels. Since cortisol inhibits sst_2 expression, these suppressive effects

might subsequently be (partially) abrogated. In this relative hypocortisolemic state, enhanced ACTH inhibition via restored sst_2 expression becomes suggestive. Therefore, SOM230 may be able to lower ACTH levels in Cushing's disease even more, because it could now function via both sst_5 and sst_2 receptor subtypes. Nevertheless, it should be kept in mind that this tantalizing hypothesis needs further studies to confirm its rationale. These studies should be confirmed in primary cultures of rodent corticotroph cells and in living animals as well, before a a well designed clinical trial in patients with Cushing's disease can be performed. Internalization of receptor-ligand complexes has been shown to play a role in desensitization (53), leading to tachyphylaxis of the inhibitory effect of sst_2-preferring analogs on hormone secretion in a subgroup of neuroendocrine tumours (54, 55). Prolonged treatment of AtT-20 cells with SRIF-14 results in desensitization of its inhibitory effect on ACTH secretion and cAMP formation (56), and prolonged exposure of AtT-20 cells to SRIF-14 and SRIF-28 has been shown to down-regulate SRIF-14 receptors (57). Interestingly, we recently observed that prolonged SOM230 treatment, but not OCT, inhibited basal ACTH release both from primary cultures of ACTH-producing pituitary and from AtT-20 cells (23) suggesting that sst_2 and sst_5 appear to respond differently after continued ligand activation. The ability of the sst_5-preferring analogs to continue to suppress ACTH levels, independent of prior 72 h SRIF-analog exposure and the decreased efficacy of OCT, supports other data that sst_2 desensitizes on continued ligand activation and suggests that the sst_5 receptor may be more resistant to desensitization. It has already been demonstrated that sst_5 receptors are rapidly recycled and restored from intracellular storage after agonist activation, which might protect this particular receptor from long-term down-regulation (58). In addition, it was recently demonstrated in live transfected AtT-20 cells that only fluorescein protein (FP-) tagged sst_2 subtype, but not FP-sst_5, internalized upon ligand activation (59). The FP-sst_5 subtype remained localized to the membrane during treatment with either a sst_5 preferring agonist, a sst_{2+5} biselective agonist, SRIF-14 or SRIF-28. In summary, this study demonstrates that sst_5 receptors display intriguing functional properties in regulating ACTH release in mouse corticotroph

tumour cells. Moreover, the recent observation that in sst_5 knockout mice serum ACTH and cortisol levels were elevated compared with wild type mice supports the concept that sst_5 receptors are important in the regulation of ACTH release in mice (60). Based on the potent suppressive effects on ACTH release by sst_5 preferring analogs, the relative resistance of sst_5 expression and action to DEX suppression as well as to prolonged SRIF-analog exposure, and our recent observation that sst_5 is the predominant sst expressed in human corticotroph adenomas (23), we propose that sst_5 may become a new therapeutic target for the control of ACTH and cortisol hypersecretion in untreated patients with pituitary dependent Cushing's disease.

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Chapter VI

The Role of Novel Somatostatin Analogs in the Treatment of Neuroendocrine Tumours

Chapter VI-1

Novel subtype specific and universal somatostatin analogues: Clinical potential and pitfalls

Adapted from

Curr Pharm Des. 2005, 11 (12):1573-92

Recent developments of novel somatostatin analogs

Somatostatin (SRIF, **1**) inhibits a variety of physiological processes. Initially, its possible therapeutic value in clinical conditions involving hyperfunction of several (endocrine) organ systems seemed very promising. However, the multiple simultaneous effects of SRIF in different organs, the need for intravenous administration (a half-life, $T_{1/2}$, in the circulation of less than three minutes), and the post-infusion hypersecretion of hormones (GH, insulin and glucagon) considerably hampered the initial enthusiasm for its clinical use (1).

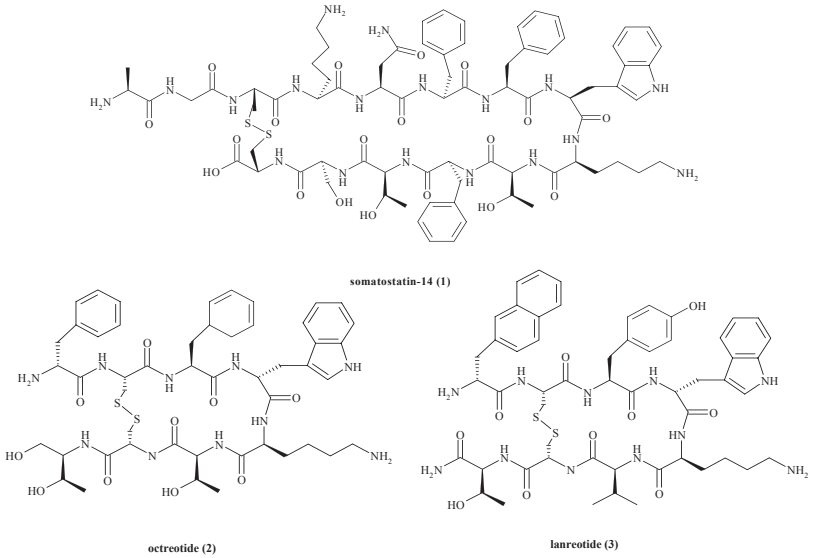

Figure 1. Structures of the natural somatostatin peptide SRIF and two current clinically available SRIF-analogues octreotide and lanreotide. Compounds are referred to using bold numbers in the main text

Attempts have been made to synthesize SRIF-analogs that do not have these disadvantages. To design a more stable peptide derivative, one needs to strengthen the metabolic resistance of the cleavage sequences of the native peptide. In the case of SRIF, the most critical cleavage can occur at amino acid Tryptophan on position 8, because such a rupture leads to completely inactive fragments. Essential structural features of SRIF include the β-turn comprising amino acids; phenylalanine[7] (Phe), tryptophan[8] (Trp), lysine[9] (Lys) and threonine[10] (Thr) as well as the cysteine-cys-

teine (Cys-Cys) bridge between position 3 and terminal position 14 (2, 3).

SRIF-receptor agonists

The clinically available short synthetic SRIF-analogs octreotide (**2**) and lanreotide (**3**), encapturing a Cys-Cys bridge, contain the β-turn which has been stabilized by incorporation of D-Trp (Fig. **1**). Both peptides display selective high-affinity binding to sst_2 along with lower affinity binding to sst_3 and sst_5 and (almost) no binding to sst_1 and sst_4. Pharmacokinetic data on the metabolism and elimination of octreotide and lanreotide, compared to SRIF, were found to show enhanced metabolic stability ($T_{1/2}$ of 2 h and less than 1h, respectively), a small volume of distribution and low clearance, all resulting in a longer duration of exposure and consequently a longer lasting biological activity. Furthermore, rebound hypersecretion of hormones does not occur, making these analogs feasible for clinical use (4, 5). BioMeasure introduced several peptide sst subtype specific SRIF-analogs, which were predominantly directed towards sst_2 (BIM-23190, BIM-23197) and sst_5 (BIM-23052, BIM-23268 (**4**) (6-8) (Table I). Whereas the sst_2 specific compounds are classical cyclic analogs, the sst_5 specific analog BIM-23052 is a linear peptide, and all three were assembled by solid- or liquid-phase synthesis using conventional fluorenylmethyloxycarbonyl methods. BIM-23268 (**4**) harbours a unique structure and differs from other cyclic octapeptides in that the characteristic disulphide bridge of this cyclic peptide begins and ends at the NH_2 and COOH terminals, rather than positions 2 and 7 (Fig. **2**). These sst_2 and sst_5 analogs appeared highly potent in suppressing human GH and TSH in cultures of human fetal pituitaries, whereas PRL secretion in fetal human lactotroph cultures was reduced via sst_2 selective compounds alone. BIM-23926 and BIM-23745, both proposed sst_1 specific agonists have been introduced *in vitro* as well, but no insights have been released about their pharmacological development (9-11). However, only biodistribution of BIM-23190 and BIM-23197 was determined in rats (12). Even though these two analogs seemed to achieve longer *in vivo* stability, higher plasma and tissue levels compared to lanreotide (**3**), these BIM compounds have not been used in a clinical setting yet. The main reason seems to be a lack of additivity of

these BIM analogs regarding lanreotide and octreotide, as their sst binding affinity profile is comparable and further clinical efficacy would not be expected. Another explanation might be the new insights into SRIF-receptor physiology, as already discussed in the previous section. As a consequence of the demonstrated molecular cross-talk not only between different members of the sst subfamily but also between different related

Table I. Binding selectivity of SRIF-agonists to all sst subtypes.

Agonist	Receptor subtype binding affinity (IC_{50} in nM)				
	sst_1	sst_2	sst_3	sst_4	sst_5
SS-14 (**1**)	2.3	0.2	1.4	1.8	0.9
octreotide (**2**)	>1000	0.6	34.5	>1000	7
lanreotide (**3**)	>1000	0.8	107	>1000	5.2
BIM-23926	3.6	>1000	>1000	833	788
BIM-23745	42	>1000	>1000	>1000	>1000
BIM-23197	>1000	0.2	26.8	>1000	9.8
BIM-23268 (**4**)	18.4	15.1	61.6	16.3	0.4
BIM-23244	>1000	0.3	133	>1000	0.2
BIM-23A387	293	0.1	77	>1000	>1000
β-peptide (**5**)[a]	4.73	4.48	4.85	7.83	4.73
compound **6**	>1000	807	750	0.84	633
γ-peptide (**7**)[a]	6.26	5.17	6.00	5.92	5.87
SOM230 (**8**)	9.3	1	1.5	>100	0.16
PTR-3173 (**9**)	>1000	3	>100	7	6
KE108 (**10**)	2.6	0.9	1.5	1.6	0.65
cyclic urea (**11**)[b]	n.i.	8.5	>1000	n.i.	>1000
L-797,591[b]	1.4	>1000	>1000	170	>1000
L-779,976[b]	>1000	0.05	729	310	>1000
L-796,778[b]	>1000	>1000	24	>1000	>1000
L-803,087[b]	199	>1000	>1000	0.7	>1000
L-817,818 (**12**)[b]	3.3	52	64	82	0.4

Data are derived from Refs. 6-11, 16-17, 19-20, 22-25 and 27. n.i., not investigated.
a Values represent pKD values (–log10 concentration constant).
b Values represent Ki values in nM.

G-protein coupled receptor families, the BioMeasure company recently developed two SRIF-analogs, of which no pharmacological insights in

the chemical structure of these two compounds have been published yet. BIM-23244 is the new bispecific analog with high affinity and selectivity for both sst_2 and sst_5 (13) (Table I). This peptide can activate both receptors, and because of the heterogeneous expression of sst_2 and sst_5 in neuroendocrine tumours, this analog has the potency to achieve a better control of hormonal hypersecretion. The second compound, BIM23A387, contains the ability to bind in nmol/L range both to sst_2 and the D2 receptor (14) (Table I). This chimeric SRIF-Dopamine ("dopastatin") hybrid molecule, clearly provides a tool to further elucidate and unravel the oligo

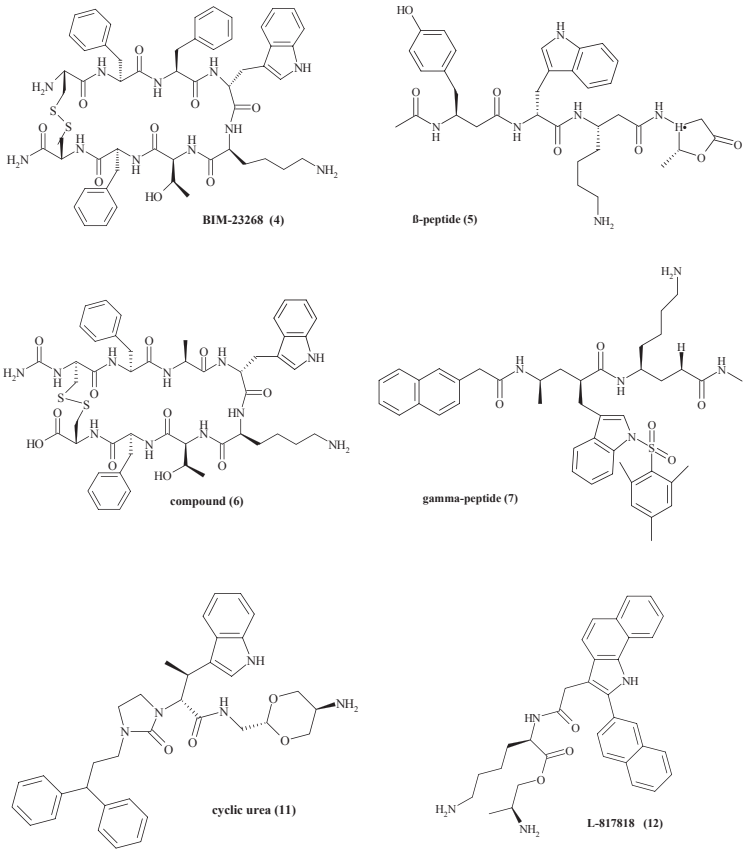

Figure 2. Structures of somatostatin receptor subtype specific agonists. Compounds are referred to using bold numbers in the main text.

meric interactions among different members of the GPCR family (15). As

will be outlined in the next chapter, the practical consequences of these new compounds, demonstrating possible novel receptor complexes with enhanced functional activity, have recently been demonstrated *in vitro*. Another promising new compound series are the β-peptides, still encapturing the pharmacophore D-Trp/L-Lys, which are easily adapted to secondary structures and have the advantage of total stability against proteolytic degradation. Nunn and coworkers demonstrated this strategy indeed to have potentials, when they characterised the first β-peptide sst_4 selective compounds (**5**) (16). So far, these compounds displayed only moderate affinities in recombinant human and mouse sst_4 receptors (Table I). More recently, based on rather simple amino acid substitutions, the group of Reubi launched several sst_4 specific analogs as well. On the basis of their observations that amino acid position 7 in SRIF is important for selective sst_4 binding, which was confirmed by alanine scanning of SRIF (17), they used their ODT-8 (H-c[Cys-Phe-Phe-DTrp-Lys-Thr-Phe-Cys]-OH) octapeptide scaffold as a template for optimizing specific binding and selectivity for sst_4 (18). When the L-Trp isomer, suggested to be more selective to sst_4 whereas D-Trp tends to increase potency to all receptors, was incorporated at position 8 and alanine at position 7, an unexpected high sst_4 affinity and selectivity was observed (compound **6**). Tyrosine substitution at the N-terminus of the compound did not negatively impact either affinity or selectivity and radiolabeling with ^{125}I demonstrated sst_4 specific binding as well (19). Although the sst_4 subtype is poorly expressed in human tissues, these compounds still could serve as a tool to elucidate its (patho-) physiological role and distribution throughout the human body. Moreover, as an extension of the β-peptide approach, a series of simple γ-dipeptide (**7**) derivatives were synthesized for sst binding affinity (20). These derivatives have the Trp side chain in the γ2-position of the first amino acid and the Lys moiety at the γ4-position of the second amino acid. So far, one of the synthesized γ-dipeptides showed only very moderate (submicromolar) affinity for sst_1 and sst_3. Apparently, a 14 amino acid cyclic disulphide hormone, can be mimicked by a simple, designed, low molecular weight, open-chain γ-dipeptide derivative that contains only three amide bonds and they promise a potential of γ-dipep

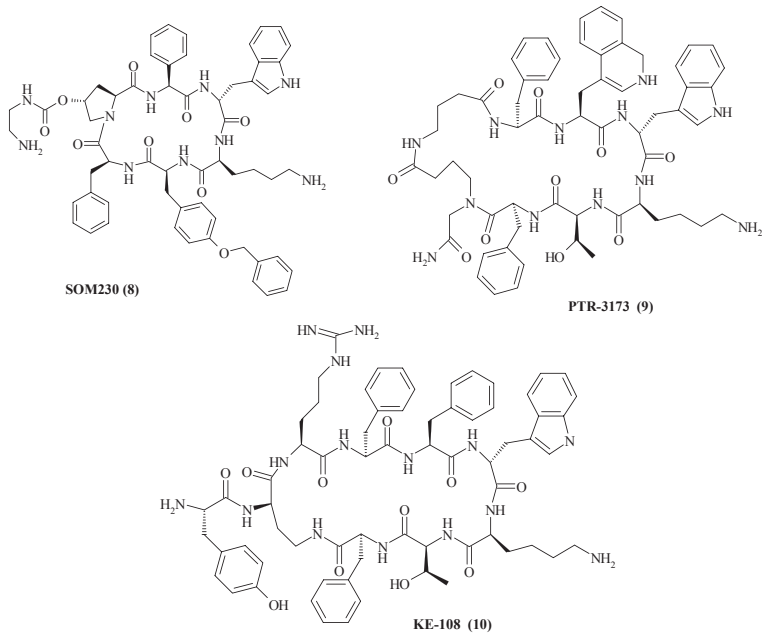

Figure 3. Structures of universal somatostatin receptor analogues. Compounds are referred to using bold numbers in the main text.

tides for the development of peptidase-resistant peptidomimetic drugs. Unlike most previous studies to develop subtype-selective SRIF-analogs, more universal binding SRIF-analogs have been characterised and introduced recently as well (Fig. **3**). The search for SRIF-analogs with a high affinity binding profile to all five subtypes was only started a few years ago, when it became clear that differential expression of the five sst subtypes in pathological SRIF targets hampers successful treatment with the current clinically available sst_2 preferring SRIF-analogs. Therefore, universal SRIF-analogs could offer a therapeutic advantage in the treatment of tumours expressing multiple sst subtypes. In this respect, the Novartis company has recently introduced SOM230 (**8**) (17). By using alanine scanning technology, the essential functional groups of the SRIF peptide, comprising the β-turn, responsible for the high affinity to all five sst subtypes were detected (Table I). The incorporation of Lys^4 in the form of a novel basic trans-(L)-hydroxyproline aminoethyl-urethane extension, phenyglycine, O-benzyl-tyrosine, and D-Trp to corresponding positions

resulted in SOM230 (**8**). This stable cyclohexapeptide binds with a high affinity to sst_1, sst_2, sst_3 and sst_5, and with low affinity to sst_4. In rats, dogs and monkeys SOM230 potently and dose-dependently decreases GH and Insulin-like Growth Factor I (IGF-I) levels, which appeared more efficacious compared to octreotide (21, 22). No desensitization of the suppressing effect of SOM230 on IGF-I levels was observed, which is in contrast to what has been seen with octreotide in rodents. Potential explanations for this marked IGF-I lowering effects of SOM230, compared to octreotide, include: 1) a more pronounced GH-dependent lowering of IGF-I; and 2) a postulated direct inhibitory effect of SOM230, independent of pituitary effects and exerted at the level of the liver (21). Finally, an additional very favourable characteristic of SOM230 is a half-life of nearly 24 hours. Another long acting SRIF-analog, (c[GABA-Phe-Trp-D-Trp-Lys-Thr-Phe-GlyC3-NH2], PTR-3173 (**9**), has been recently described (23). PTR-3173 (Fig. **3**) is a novel cyclic-backbone based SRIF-analog, derived from libraries of backbone cyclic SRIF-analogs which were tested following their synthesis by solid phase, multiple parallel synthesis, using F-moc chemistry. The backbone cycling building, possessing *in vivo* endocrine efficacy and selectivity, were protected on their ω-carboxy by allyl/alloc protecting group, which was removed before on resin cyclization. This peptide showed 1000- and 10,000 fold more potent *in vivo* inhibition of GH release compared with inhibition of insulin and glucagon secretion, respectively. PTR-3173 bound with high affinity for sst_2, sst_4, and sst_5 and is reported to be the first SRIF-analog that demonstrates selective *in vivo* (in rats) inhibition between GH and insulin release (Table I). Also, Reubi and coworkers presented, based on a nonapeptide scaffold, another universal SRIF-analog, [Tyr^0-(cyclo-D-Dab-Arg-Phe-Phe-D-Trp-Lys-Thr-Phe), KE108 (**10**)], which compared to SRIF-28, encaptured equivalent affinity for sst_1 but 2 to 4 times higher affinity for $sst_{2\text{-}5}$ (24) (Table I). Moreover, KE108 (Fig. **3**) showed agonistic properties for the five sst's, which was demonstrated by inhibition of forskolin stimulated cAMP production in CHO-K1 cells, stably expressing all five sst subtypes. Again, these inhibitory properties of KE108 (its structural determinants for the universal profile are at present unknown) were within the

same levels and concentrations as SRIF-28. Two additional features of this peptide seem interesting as well. First, during a 24 h incubation of the compound in human serum, HPLC detection revealed a major peak corresponding to the intact peptide for several hours. Second, because of its Tyr in position zero, this universal SRIF-analog can be easily labelled with radioactive iodine and could be used as a tracer *in vitro* and *in vivo* to identify tissues expressing SRIF receptors other than sst_2. Therefore, the *in vivo* characterization of KE108, such as its stability, metabolism, biodistribution and toxicology, have to be the next necessary steps before clinical applications, i.e. phase I clinical trials, can be considered. During the late 90s, the first data upon non-peptide SRIF analogs were published, possibly not only providing new tools to unravel the physiological role of the individual sst subtypes but it might eventually result in developing an orally active agent capable of crossing the blood-brain barrier as well. Rohrer and coworkers combined an integrated approach of combinatorial chemistry and high-throughput receptor binding techniques to rapidly identify subtype-selective compounds (25). A cyclic hexapeptide SRIF agonist (L-363,377) was used as a probe, in which the β-turn forming amino acids were given priority in their search. The compound L-264,930 which contains a tripartite in structure with an aromatic moiety, a tryptophan moiety and a diamine moiety, appeared to be the most potent and became the original lead structure (26), to which the five non-peptide sst selective analogs were related to (Table I). An important pharmacokinetic improvement was achieved by urea backbone cyclization of acyclic urea precursors, which resulted in a non-peptide sst_2 agonist (**11**) with high affinity for sst_2 and high bioavailability in rats (27). Currently, no recent new non-peptide SRIF-agonists, displaying high affinity to sst subtypes, have been identified. Therefore, the five subtype selective agonists, L-797,591 (sst_1), L-779,976 (sst_2), L-796,778 (sst_3), L-803,087 (sst_4) and L-817,818 (**12**, sst_5) (Fig. **2**), are still frequently used for defining the physiological functions of each individual sst subtype.

SRIF-receptor antagonists

However, in order to elucidate conclusively the physiological effects of

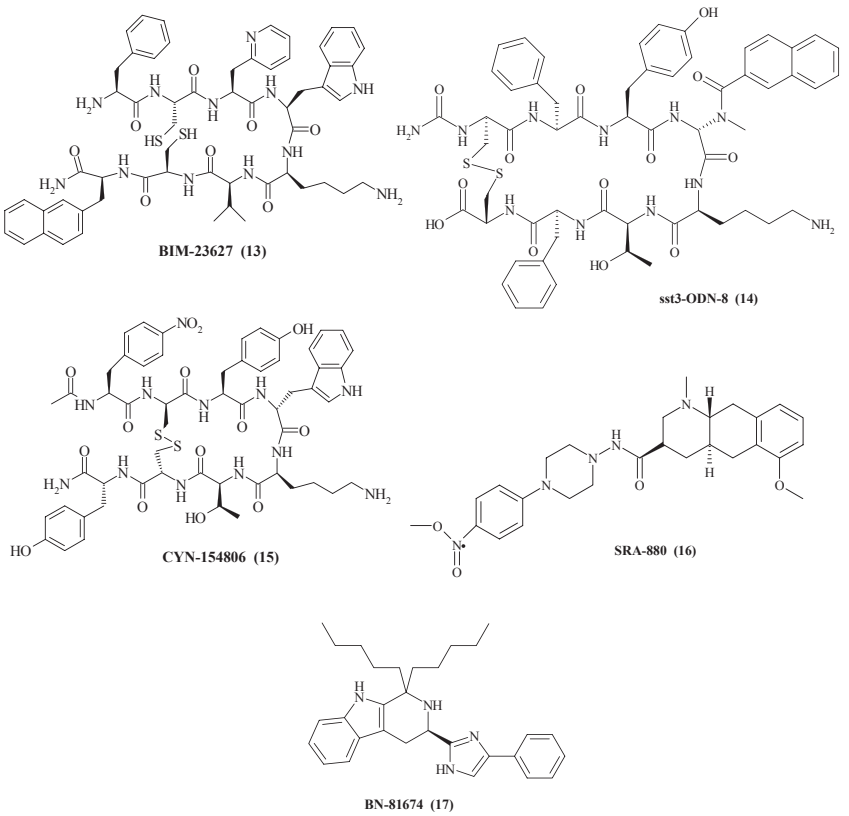

Figure 4. Structures of somatostatin receptor antagonists. Compounds are referred to using bold numbers in the main text.

individual sst subtypes, the combined use of both agonists and antagonists (Fig. **4**) seems interesting as well. BIM-23454 and BIM-23627 (**13**), both sst_2 specific open-chain octapeptide antagonists, as well as BIM-23056, a sst_5 specific linear octapeptide specific antagonist, all three incorporated with an aromatic β-naphtoyl moiety, have been released by BioMeasure in order to retrieve answers within the sst receptor physiology (28). Their role will be discussed in the next section.A family of octapeptide derivatives of SRIF-cyclized via a disulphide bridge that showed high affinity and selectivity for the human sst_3 was identified (18). Binding affinity for sst_3 appeared equal to that of SRIF-28, whereas affinity towards the other 4 receptor subtypes appeared 1000-fold less than SRIF-28. Compound

sst_3-ODN-8 (**14**), in which the conformation-restricting N-methyl-amino-2-naphtoyl aminoglycine replaced Trp in the β-turn, potently reversed SRIF induced forskolin-stimulated cAMP production as well as SRIF-induced stimulation of phospholipase C activity, making this peptide a specific and selective sst_3 antagonist (Table II) (18). Since the tyrosine at position 7 could be radioiodinated as well, this antagonist seems of special interest regarding the study of sst_3-mediated (patho-) physiological conditions in areas of the lymphoreticular system and cell growth (apoptosis). CYN 154806 (**15**), another cyclic octapeptide first reported in 1996, displays nanomolar affinity for sst_2, and L-Tyr^8 and D-Tyr^8 isoforms have been shown to possess antagonism at sst_2 receptors (Table II) (29, 30). Recently, it has been shown that both isoforms of this antagonist also displayed high affinity for sst_5 and agonist like properties as well. In

Table II. Binding selectivity of SRIF-antagonists to all sst subtypes.

Antagonist	Receptor subtype binding affinity (IC50 in nM)				
	sst_1	sst_2	sst_3	sst_4	sst_5
BIM-23454	>1000	31	50.5	301	139
BIM-23627 (**13**)	>1000	6.4	44	423	86.5
BIM-23056	337	132	177	234	12.1
sst_3-ODN-8 (**14**)	>10.000	>10.000	6.7	>10.000	>10.000
CYN 154806 (**15**)[a]	5.7	8.14	6.23	6.5	7.65
SRA-880 (**16**)[a]	8.1	4.7	5.4	5.3	5.9
BN-81674 (**17**)[b]	>10.000	>10.000	0.92	>10.000	>10.000

Data are derived from Refs. 6, 18, 28-29, 31 and 33-34.
a Values represent pKD values (–log10 concentration constant).
b Values represent Ki values in nM.

CHO cells transfected with sst_2, concentration-dependent inhibition of forskolin stimulated adenylyl cyclase activity was found (31). Therefore, CYN 154806 seems not totally sst subtype selective and it harbours intrinsic activity at sst_2 as well, and should be used with caution as an sst_2 selective antagonist. Agonist properties of other putative small-molecule sst_2 antagonists has been demonstrated as well (32), which might hamper the use of these SRIF-tools in the field of SRIF-physiology. SRA-880 (**16**) is a novel non-peptide sst_1 antagonist (IC_{50}= 9.1 nM) based

on a octahydrobenzo[g]quinoline backbone (33). SRA-880, tested *in vivo* using a mouse model, is at least active in the neuro-psychiatric field of anxiety, depression and bipolar disorders. The development of selective sst_3 non-peptide antagonists, BN-81674 (**17**) is a tetrahydro-β-carboline derivative showing a pKd value of 8.69 nM for human sst_3 receptor along with a 400-fold greater sst_3 selectivity compared to the other sst subtypes, enables functional investigation of the sst_3 regarding cell growth and apoptosis (34). Such compounds might eventually combine the advantages of oral bioavailability with various therapeutic opportunities.

Clinical potential

Somatostatin analogs in the medical treatment of hormone-secreting pituitary adenomas and GEP tumours

The clinical introduction of the long acting SRIF-analogs octreotide (**2**) and lanreotide (**3**) in the early 1980s added a new dimension to the therapy of GH-secreting pituitary adenomas (35, 36). Throughout the past two decades several clinical trials with these SRIF-analogs, administered either by subcutaneous (s.c.) or as a long-acting depot preparation by intramuscular injection, repeatedly turned out to be effective in around 60-70% of patients in controlling GH and IGF-I levels (4). However, approximately 35% of acromegalic patients appear not to be sensitive enough for this treatment, since in this group of partial responders GH levels can not be controlled to "safe" levels. In addition, notable shrinkage also occurs in some patients, based on a decrease in the size of individual pituitary tumour cells, which no longer synthesize and secrete hormones (37, 38). The efficacy of SRIF-analogs in non-GH-secreting pituitary adenomas has still to be proven in clinically non-functioning adenomas, besides the well-known expression of sst subtypes in these tumours, whereby sst_3 seems to be the predominant subtype (39). The large majority of patients with TSH-secreting pituitary adenomas respond very well to treatment with either octreotide or lanreotide, although long-term data are still limited (40, 41). Prolactin- and ACTH-secreting pituitary adenomas generally respond weakly to treatment with the current

clinically available SRIF-analogs, while in vitro experimental results show a high PRL-suppressive effect of agonists selective for sst_5 (42). In most patients with metastatic carcinoid disease and islet cell tumours, octreotide therapy also improves clinical symptoms (43). Control of flushing attacks and diarrhoea, caused by an overproduction of serotonin or tachykinin(s), was reported in 70-90% of patients with metastatic carcinoid tumours (5, 44). Results from studies also suggest a temporary stabilization of tumour growth during SRIF-analog therapy in 30-60% of patients with carcinoids or GEP tumours (45). However, as will be shortly discussed in the *Pitfalls* section, an important aspect of the long-term successful control of hormone secretion and tumour cell growth during SRIF-analog treatment is a loss of effect (tachyphylaxis). Frequently, newly developed SRIF-analogs are primarily being explored within GH-secreting pituitary adenomas (7). The significant percentage of GH-secreting pituitary tumours relatively resistant to octreotide (**2**) and lanreotide (**3**), may be explained in part by a variable tumoural expression or reduced receptor density of sst subtypes on the adenomas of these patients. Saveanu and coworkers compared the *in vivo* sensitivity of GH release for octreotide in nine acromegalic patients with the tumour mRNA expression for sst_2 and sst_5 subtypes (13). It was observed that sst_2 mRNA expression was lower and sst_5 mRNA was higher in adenomas that were partially sensitive to octreotide, compared with octreotide sensitive adenomas. In the group of partially octreotide sensitive tumours, both the sst_5-preferential analog BIM-23268 (**4**), but especially the sst_2 and sst_5 bi-specific compound BIM-23244, were quite effective in suppressing GH secretion. Indeed, enhanced suppression (73%) of GHRH stimulated GH secretion from fetal pituitary cells using combined sst_2 and sst_5 selective agonists, compared with the use of these analogs alone (32% for sst_2 and 34% for sst_5) was demonstrated. These data indicate that due to the heterogeneous expression of sst_2 and sst_5 subtypes in GH-secreting adenomas, a bispecific analog, such as BIM-23244 that can activate both receptors possibly because this bivalent ligand mediates receptor heterodimerization, may achieve a better control of GH hypersecretion of GH-producing pituitary tumours than octreotide. Also, Melmed's group

recently showed that the sst_2 antagonist BIM-23454 completely blocked GH release in primary fetal pituitary cultures treated either simultaneously with sst_2 (BIM-23197) and sst_5 agonists (BIM-23268) or with BIM-23244 (46). These data again suggested that both receptors need a functional interaction before enhanced suppression of GH release is established.Furthermore, the hybrid 'dopastatin' molecule, BIM-23A387, has an enhanced inhibitory effect on *in vitro* PRL and GH release from human pituitary adenoma cells (14). This dimer molecule appeared more potent than either sst_2 or D2 selective analogs alone and interestingly, no additivity was even found when the sst_2 and D2 selective analogs were added simultaneously to the primary cultured GH-secreting pituitary adenoma cells. This significant potency of BIM-23A387 (EC_{50} was 50 times lower than that of the individual sst_2 and D2 agonists as well), however, could not be explained on the basis of the binding affinity of the compounds for sst_2 and D2 receptors. In addition, only the combined addition of the sst_2 antagonist BIM-23454 and D2 antagonist sulpiride completely blocked the effects of BIM-23A387, indicating that signaling properties of the receptors may be dependent on having the binding requirements for each receptor within the same molecule. In contrast, performing the same kind of experiments in primary cultured human fetal pituitary and human GH/PRL secreting pituitary adenoma cells, Melmed's group did find complete blockade only after sulpiride, but not after BIM-23454 treatment (47). The observed difference between the two study groups could be explained because the latter study used BIM-23A387 at supramaximal concentrations (4 nmol/L), whereas Saveanu and coworkers used a 50% effective dose (1 pmol/L) (14). At lower doses, BIM-23A387 ligand-induced heterodimerization of sst_2 and D2 receptors may not be sufficient to saturate the chimeric molecule, so that free BIM-23A387 is still available for banding sst_2 or D2 receptors alone (47). Still, collectively these results suggest that GH suppression by BIM-23A387, is not mediated through either individual sst_2 or D2 receptors, but requires a functional interaction between the two receptors in which the D2 receptor might have a dominant role. Therefore, the intriguing *in vitro* data with both BIM-23244 and BIM-23A387, fully in line with the new insights in

SRIF-receptor physiology, indicate that processes like heterooligomerization of G-protein coupled receptors could indeed create a novel receptor with distinct functionality, that might have functional and clinical implications in the treatment of neuroendocrine tumours. Recently, using the sst_1 specific analog BIM-23926, GH and PRL secretion was significantly inhibited in primary cultures of GH/PRL-secreting pituitary adenomas (10). BIM-23745, another sst_1 specific analog, showed suppressive actions on GH release in primary cultured GH-secreting pituitary adenomas as well, even in a subgroup of adenomas that partially responded to octreotide or lanreotide (11). Since the sst_1 receptor appears monomeric upon receptor activation even if it can be recruited in heterodimer formation by an activated sst_5 (48), SRIF analogs with enhanced affinity for sst_1 may have potential as a pharmacological tool for the treatment of this type of pituitary adenoma. The latest insights in new medical treatment options for pituitary and GEP tumours come from the first clinical trial with SOM230 (**8**) in patients with active acromegaly, which has been extensively described and discussed in **Chapter III** of this thesis.

Somatostatin analog therapy in oncology

Biological and clinical effects of SRIF and SRIF-analogs

In contrast to the antisecretory properties of SRIF, its possible antiproliferative effects were documented largely through use of the long acting analogs for the treatment of hormone hypersecretion from pancreatic, intestinal, and pituitary tumours (43). It was noted that SRIF not only blocked hormone hypersecretion from these tumours but also caused variable tumour shrinkage possibly through an additional antiproliferative effect, which appeared not limited to endocrine tumours. Several preclinical data and clinical advances have brought the potential use of SRIF analogs in the treatment of patients with cancer much closer to reality. The presumed potential antiproliferative effects of SRIF and SRIF-analogs can be a result of a decrease in both tumour cell growth and tumour angiogenesis as well as an increased incidence of (cancer) cell apoptosis. As has been reviewed extensively, these inhibitory effects at the cellular level can be based on direct and indirect mechanisms (49, 50). In

general, all five sst subtypes have been shown to modulate antiproliferative second messenger pathways, such as protein tyrosine phospatases, SHP-1, SHP-2, MAP/ERK1/2 kinases and the subsequent induction of cyclin-dependent kinase inhibitors such as p21 and $p27^{Kip1}$ (51-53). Indirect mechanisms of cell growth may include inhibition of secretion of growth-promoting hormones and growth factors, i.e. IGF-I, EGF-I and PDGF. Also, SRIF inhibits tumour angiogenesis via sst_2 (54), whereby sst_3 as well might inhibit endothelial nitric oxidase and the following attenuation of nitric oxide, a second messenger that plays a pivotal role in angiogenesis (55). Evidence further suggests that SRIF may influence the immune system, since SRIF-receptors are expressed in human lymphoid organs and can regulate various immune functions as well (56-58). Finally, sst_3 and to a lesser extent sst_2 seem to be involved in mediating cell growth arrest by the induction of apoptosis in normal and tumour cells (53, 59). Still, no specific evidence of a pro-apoptotic role of SRIF-analogs in cancer has been documented so far. Despite promising in vitro data, attempts to use these analogs for therapy of human cancers have produced few beneficial effects (44). Recent trials still show disappointing results when lanreotide (**3**), vapreotide (2x) and octreotide (**2**) were administered respectively to patients with advanced hepatocellular carcinoma, advanced prostatic cancer, metastatic breast cancer and again advanced hepatocellular carcinoma (60-63). Hejna and co-workers elegantly reviewed the clinical application of SRIF-analogs in malignant diseases (64). While SRIF-analogs are highly effective in the symptomatic management of patients with neuroendocrine tumours, the antiproliferative effects of SRIF-analogs as judged by objective tumour regression has not been convincingly demonstrated. An explanation comes likely from the fact that in various cancers there is a loss of gene expression for sst_2, which is the preferred subtype of these analogs. However, the expression of sst_1 (prostate), sst_5 and sst_3 should make possible the therapy with novel SRIF-analogs. Again, SOM230 can also further help in elucidating and resolving these issues if sst_1- and sst_3-mediated antiproliferative effects might have a clinically beneficial effect. Moreover, the long-term and sustained suppression of IGF-I might also have new, important clinical

significance in the control of tumour growth in a variety of IGF-I responsive solid cancers. The promising β-casomorphin derived cyclopentapeptide SRIF-analog, Tyr-c[D-Orn-Tyr(Bzl)-Pro-Gly] (cCD-2), has been developed by conventional solution methods (65), which appears interesting especially regarding the consequences of the demonstrated

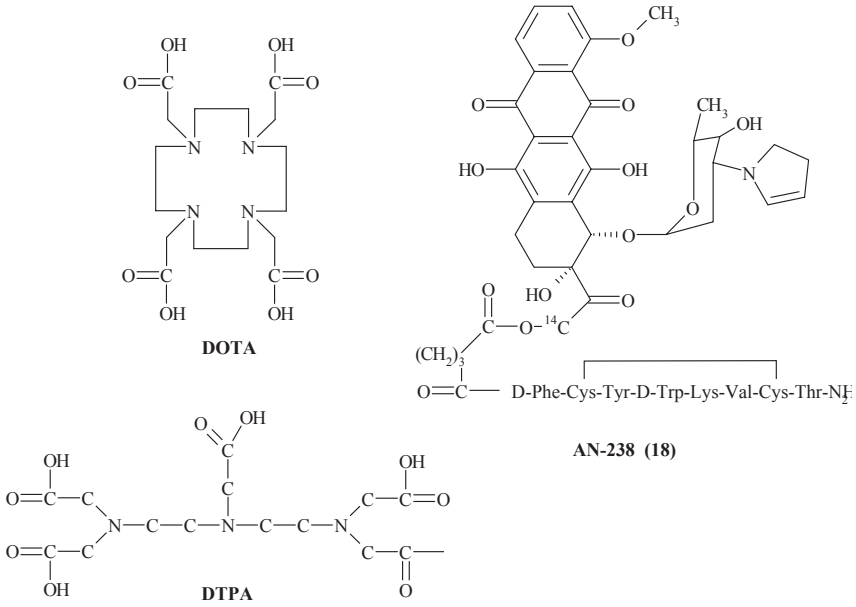

Figure 5. Structure of two frequently used chelators for radiolabeling SRIF-analogues, as well as the molecular structure of the cytotoxic hybrid SS-analogue AN-238. To achieve tight binding of therapeutically used radioisotopes, bifunctional chelators are used. In such bifunctional chelators, all coordination sites are free to bind the metal ion and the peptide is connected to the chelator at a site that does not interfere with the metal ion binding site.

molecular cross-talk between members of the SRIF- and opioid-receptor family (66). Using different human tumour cell lines, cDC-2 inhibited cell growth by activation of sst_1 or sst_2 and subsequent downstream PTP, MAPK and cyclin dependent kinase inhibitor p21 activation. The addition of specific opioid-receptor antagonists, as opioid agonists are known to decrease cell proliferation via μ-, κ-, and δ-receptors (67), did not attenuate the cell growth inhibition, indicating that only sst subtypes can be responsible. Furthermore, whereas cDC-2 itself only has a very low affinity to μ-receptors, this compound was able to increase the agonist

binding to μ-receptors *in vitro* and to potentiate the analgesic effect of the μ-receptor agonist morphine *in vivo*, indicating some kind of μ-receptor sensitization by cCD-2. However, SRIF-receptor binding affinity of cDC-2 was very moderate (IC_{50} = 250 nM), and might be too low for possible clinical use. Nevertheless, the combination of two therapeutically interesting properties, such as inhibition of tumour cell growth and stimulation of analgesic potency of morphine within the structure of a single pentapeptide might be useful in the development of new anticancer drugs.

Somatostatin-receptor targeted radio- and chemotherapy

The demonstration of the efficient internalization of receptor-ligand complexes into sst-positive cells, as already previously described, formed the basis for the concept of targeted sst-mediated chemo- or radiotherapy of sst-expressing metastatic human cancer (3, 68-71). Internalization brings the cytotoxic SRIF-analog or the radionuclide-coupled analog closer to the nucleus of the cell, resulting in prolonged cellular retention and exposure to radioactivity or cytotoxic agent. Human sst-positive tumours show a high uptake of ^{111}In-diethylenetriamine pentaacetic acid (DTPA, Fig. **5**) octreotide (Octreoscan) at sst-scintigraphy (72, 73). Recently, it was demonstrated by using an in vivo sst_2 knock-out mouse model, that the sst_2 predominantly determines uptake of both [^{111}In-DTPA^0]octreotide and [^{111}In-DTPA^0]SRIF in sst-positive organs (the ^{0} indicates that the chelator is attached to the amino acid in the first position of the peptide). A possible explanation for the observation that also uptake of the universal radioactive ligand and [^{111}In-DTPA^0]SRIF appeared only in the wild-type mice, could be that sst_2 has a modulatory effect upon sst-mediated internalization of the other sst subtypes (74). Furthermore, as soon as the success of the Octreoscan became clear, the next logical step was to label these peptides with radionuclide emitting α- or β-particles, including Auger (radiotoxicity of Auger electrons is very high if the DNA of the cell is within the particle range, probably resulting into an effect upon tumour cell proliferation) or conversion electrons, and to perform peptide receptor radionuclide therapy (PRRT). Indeed, high dosages of [^{111}In-DTPA^0]octreotide showed anti-tumour effects in vitro and in patients with

Table III. Physical characteristics of different radionuclides.

.

	$T_{1/2}$ (days	Eβ (keV)	Range in tissue (μm)	Advantage
^{111}In	2.8	(only γ)	0.02-0.10	emits Auger electrons
^{90}Y	2.6	2,270	4-12*10^3	high maximum energy; suitable for large tumours
^{177}Lu	6.7	500	0.5-2*10^3	γ-emission (scintigraphy & dosimetry); high tumoural uptake; low renal toxicity; radiological bystander effect; suitable for smaller tumours
^{64}Cu	0.5	655 (β^+) 573 (β^-)	1-2*10^3	effective accumulation in nucleus and mitochondria; radiological bystander effect; therapy (β^+) and diagnostics(β^-)

Data have been documented from Refs. 76, 82, 87 and 104.

neuroendocrine tumours (75, 76). The disadvantage of ^{111}In for PRRT is the short particle range and consequently small tissue penetration. Therefore, other radionuclides, like ^{90}Y and ^{177}Lu (Table III), were coupled to tetra-azacyclododecane tetracetic acid (DOTA) conjugated SRIF-analogs (Fig. **5**), such as [$DOTA^0$,Tyr^3]octreotide and [$DOTA^0$,Tyr^3]octreotate (in which the C-terminal threoninol is replaced with threonine) (77). ^{90}Y is a β-particle emitter, has a mean range of several millimetres in tissue, and coupled to [$DOTA^0$,Tyr^3]octreotide (^{90}Y-SMT) induces partial remissions in 10-25% of patients with GEP tumours combined with an improvement in clinical status (78, 79). ^{177}Lu is a β-particle emitter with a mean tissue range of approximately 1mm, but it is also a γ-emitter that enables visualisation with a gamma camera and thus tumour dosimetry and staging. Pharmacodynamic properties of [^{177}Lu-$DOTA^0$,Tyr^3]octreotate show, compared to [^{111}In-$DTPA^0$]octreotide and [^{90}Y-$DOTA^0$,Tyr^3]octreotide, a 3- to 4-fold higher tumoural uptake of radioactivity and a 9-fold increase in sst_2 affinity, respectively (80). Also, labelled with ^{177}Lu, this compound was very successful in achieving tumour regression and survival in a pancreatic tumour bearing rat model (81). Because of these ad-

vantages, a study was conducted to compare this radiolabeled compound to [^{111}In-DTPA^{0}]octreotide in patients with sst-positive tumours. Kwekkeboom and coworkers found that the uptake of radioactivity, expressed as a percentage of the injected dose [^{177}Lu-DOTA^{0},Tyr^{3}]octreotate, was comparable to that after [^{111}In-DTPA^{0}]octreotide for kidneys, spleen and liver, but was three- to fourfold higher for four of the five tumours (82). They concluded that [^{177}Lu-DOTA^{0,} Tyr^{3}]octreotate potentially represents an important improvement because of (a) the higher absorbed doses that can be delivered to most tumours with about the equal doses to potentially dose-limiting organs and (b) the lower tissue penetration range of ^{177}Lu as compared to ^{90}Y, which may be especially important for small tumours. Recently, the first data on the anti-tumoural effects of ^{177}Lu-octreotate therapy in 35 patients with GEP tumours have been described (83). The investigators observed tumour shrinkage in 38% of patients, but this could be an underestimation because only 46% of included patients showed signs of progressive disease (as this percentage was around 80% in studies with the former mentioned radiolabeled SRIF-analogs), whereby it is well known that rapidly growing tumours are the most sensitive ones to PRRT or chemotherapy. Still, long-term efficicacy needs to be determined, but this study provokes to treat patients with ^{177}Lu-octreotate not only when progressive disease has been diagnosed but also already during an early stage of metastatic disease, as in this last group of patients tumour load appeared more frequently limited. Finally, combination therapy with ^{90}Y-labelled and ^{177}-Lu labelled octreotate, tackling larger and smaller tumours, respectively, may be tried in the near future as well (84, 85). This might hopefully result into better clinical responses. These promising results have triggered researchers to synthesize and test new SRIF-analogs labelled with various radiometals, such as ^{64}Cu (Table III) (86). When this radiometal was labelled to tetraazacyclotetradecane-octeotide (^{64}Cu-TETA-octreotide), an increase of ^{64}Cu localization to the cell nucleus and mitochondria after the addition of ^{64}Cu-TETA-octreotide to intact AR42J rat pancreatic tumour cells over time was observed (87). These data indicate that still considerable progress is being made, which should eventually result in better non-invasive strategies in the treatment

of GEP tumours and cancer. Like targeted radiotherapy, SRIF receptor-targeted chemotherapy represent an appealing approach to the treatment of sst expressing tumours. By synthesizing conjugates of SRIF-analogs and cytotoxic drugs (such as methotrexate or doxorubicin), selective accumulation of cytotoxic radicals in sst positive tumour cells would be possible (88, 89). The adverse reactions in patients with advanced metastatic tumours treated with chemotherapeutic agents are caused by the severe toxicity of these agents to normal cells (90). Experimental studies have actually shown that these derivatives are indeed less toxic and seem more effective than the parent cytotoxic drugs in inhibiting tumour growth *in vitro* and *in vivo* in preclinical tumour models. The 'magic bullet' approach of developing targeted hybrids directed against sst was studied extensively by Schally and coworkers, who synthesized AN-238 (**18**, Fig. **5**), made by coupling one molecule 2-pyrrolino-DOX-14-o-hemiglutarate to the NH_2 terminus of [lys(fluorenylmethoxycarbonyl)^5]RC-121, followed by deprotection and purification (89). Treatment with AN-238 (**18**) in various human experimental tumours such as prostate, breast, ovarian, renal, brain, lung, pancreatic and colorectal cancer appeared to induce an average growth inhibition of more than 50-70% (88, 91). Recently, three nude mice *in vivo* gastric carcinoma models confirmed the potency of AN-238, which appeared correlated to the expression of sst_2 and sst_5 as well (92). The next step in the further development of this strategy is under way, since clinical trials with this compound are being planned for the near future.

Pitfalls

General considerations

The success of a newly developed SRIF-analog is predominantly based upon its bioavailability or metabolic stability, which is the main therapeutic limitation of SRIF itself ($T_{1/2}$ 2-3 min). Therefore, short-chain peptides can be built, which are metabolically stable, typically showing selectivity for one (or two) of the sst subtypes (for example octreotide (**2**); $T_{1/2}$ 2 hrs). Interestingly, these reduced sized SRIF-analogs can be further structurally modified ultimately leading to (near) universal binding to sst subtypes (for example SOM230 (**8**); $T_{1/2}$ nearly 24 hrs). Another

strategy involves characterizing larger SRIF-analogs that bind to the majority of sst subtypes, but requires metabolic stabilization through subsequent chemical modification involving the incorporation of D-amino acids or N-methylated amino acids (for example KE108 (**10**); $T_{1/2}$ not known yet). Moreover, mixing octreotide with microspheres of biodegradable glucose polymers, made it possible to deliver therapeutic octreotide levels for at least 28 days after intramuscular injection (initial s.c. administration was 3 times a day) (93, 94). Also, lanreotide (**3**) is prepared in microspheres of biodegradable lactide/glycolide copolymers as well and is administered by i.m. injection every 7-14 days, whereas the slow-release s.c. autogel formulation is active for 28 days (95). Apart from differences in the affinity profiles of unlabelled SRIF-analogs due to structural differences, whereby nanomolar affinity is strongly preferred regarding future clinical possibilities, radiolabeling or coupling of chemotherapeutic compounds of such analogs has major effects on binding affinity for the different human sst subtypes as well. Several characteristics of SRIF-analogs developed for radiotherapy, such as small structural modifications, chelator substitution, or type of radioisotope, considerably affect binding affinity which will have major implications for their efficacy and subsequent potentials for clinical use (76, 80).

Side-effects

The current sst_2-preferring SRIF octapeptide analogs are generally well tolerated and well documented. The most common side effects are gastrointestinal symptoms such as diarrhea, abdominal discomfort, and nausea. Early side effects occur in approximately 50% of patients but improve within 10-14 days, probably as a consequence of local adaptation within the gastrointestinal tract, and generally persist in <10% of patients. During long-term therapy, gall-stone development has been reported in 20-30% of patients and can be managed similar to gallstones in the general population (95). As SRIF and SRIF-analogs modulate the secretion of insulin and glucagon combined with sst subtype expression within the endocrine pancreas, reduced glucose tolerance and even overt hyperglycaemia were initially expected during long-term therapy. On the con-

trary, the effects on glucose homeostasis appeared to be minor and a mild deterioration occurred only in those without impaired glucose tolerance (5). Still, the proposed predominant role of sst_5 in mediating suppression of insulin secretion from rodent β-cells, could be a possible pitfall for newly developed sst_5 specific analogs as well as for universal SRIF-analogs (96, 97). Indeed, compared to octreotide (**2**), similar elevations of glucose concentrations were observed after SOM230 (**8**) administration in acromegalic patients (98). However, the elevated glucose levels seem not be caused by an inhibitory action on insulin release by SOM230. No data regarding the effects of SOM230 and octreotide on glucagon levels in acromegalics have been analyzed. At present, the mechanism of this transient increase in glucose levels remains uncertain. On the basis of the SOM230 and octreotide affinity profiles for sst_2 and sst_5, it seems unlikely that octreotide, binding 40-fold less to sst_5 compared to SOM230, would exert such a strong and long lasting insulin inhibition via sst_5 subtype whereas SOM230 treatment resulted in barely any inhibition. Therefore, these opposed effects of octreotide and SOM230 on insulin levels, suggest a role for sst_2 subtype in regulating human insulin secretion. Support for this hypothesis comes from recent experiments performed with isolated perfused human pancreas tissues, which showed inhibitory effects on insulin secretion when treated with octreotide or a specific sst_2 agonist in physiological concentrations, while an sst_5 agonist only inhibited insulin secretion at pharmacological doses (99). With immunoneutralization of endogenous somatostatin, the sst_2 agonist was still specific for inhibiting insulin secretion, while the sst_5 agonist had no significant effect on insulin secretion, suggesting again that sst_2 could play a role in regulating insulin secretion. Still, in cynomolgus monkeys, insulin, glucagon and glucose levels remained unchanged during seven days of high-dose infusion with SOM230. Furthermore, during an 18-week treatment with pharmacological doses of SOM230 plasma glucose levels were not changed, indicating that SOM230 is well tolerated in rats and monkeys with regard to glucose homeostasis (22). Nevertheless, the variable expression of sst subtypes throughout the body and their role in normal physiology, forms a potential pitfall as complete selectivity of newly de-

veloped SRIF-analogs towards pathological processes seems to be elusive.

Tachyphylaxis/Resistance

Octreotide controls hormone secretion effectively in most acromegalics for many years, and escape from therapy has not been observed. In striking contrast, the initial rapid improvement of clinical symptoms in the first weeks to months of SRIF-analog therapy in patients with GEP tumours gradually escapes, in spite of a an increase in the dose administered (45). The underlying mechanisms for the observed difference in developing tachyphylaxis to SRIF-analog treatment between GH-secreting pituitary adenomas on the one hand, and other types of sst-positive tumours have not been elucidated yet, but could involve the differential expression of sst subtypes, a tissue-specific desensitization, and/or homo-heterologous down-regulation of sst-subtypes, or alternatively, tissue-specific upregulation of SRIF-analog responsive sst subtypes by prolonged agonist treatment resulting in continued responsiveness. This has recently been thoroughly and extensively reviewed (68). Furthermore, as much of the current (pre-) clinical knowledge about tachyphylaxis is built upon sst_2-mediated mechanisms and sst_2-preferring analogs, possible difficulties regarding the behaviour of other sst subtypes in this matter, could come up during the further development of new non-sst_2 specific SRIF-analogs. The antiproliferative effects of SRIF-analogs as judged by objective tumour regression, however, are not likely to be promising. While activity has initially been claimed in almost all tumour entities investigated so far, the review by Hejna and co-workers has clearly shown that most series have been performed with a non-randomized approach in patients with highly disseminated disease, and the results reported are not consistent (64). Interpretation of the observed results is further complicated because sst status of the included patients is hard to obtain, making it difficult to judge whether the activity seen in some series of patients is due to a receptor-ligand interaction, indirect effects such as decreasing various growth factors or simply reflects an improvement of patients' well being due to suppression of paraneoplastic syndromes. Fur-

thermore, the discrepancy between the promising *in vitro* data and poor *in vivo* response could be provided by three other possible explanations (44): (1) most human cancers comprise a mixture of stromal tissue and different clones of epithelial tumour cells that no uniformly express sst. This contrasts sharply to experimental mostly monoclonal tumour models in animals, which, in most instances, homogeneously express sst on all tumour cells; (2) sst expression in parts of breast, prostate and colonic cancers often indicates loss of differentiation of the tumours. In general, these undifferentiated tumours with neuroendocrine cell differentiation have a poor prognosis at that stage of development. As discussed already, the synthesis of new bivalent peptides (15) and subsequent concomitant expression of several different peptide receptors in neuroendocrine tumours (100), provide promising perspectives to eventually tackle these difficulties in the treatment of neuroendocrine and malignant tumours; and (3) because of the nature of new clinical trials in oncology, often it is mainly those patients who are late in the onset of their disease who are included in the studies. In addition, there is preliminary evidence that chemotherapy might decrease the number of sst. Should this observation be verified *in vivo*, this would have widespread consequences for further planning of therapeutical trials to rule out negative (schedule-dependent) interaction between chemotherapy and SRIF-analogs.

Toxicity

Using the radiolabeled SRIF-analogs, critical organs for PRRT, such as [^{111}In-DTPA^{0}]octreotide, [^{90}Y-DOTA^{0}, Tyr^{3}]octreotide and [^{177}Lu-DOTA^{0}, tyr^{3}]octreotate, are the kidneys and bone marrow. The corresponding maximal tolerated doses for external radiation of these tissues are 23 and 2 Gy, respectively (76). Using ^{90}Y-DOTA^{0}, Tyr^{3}]octreotide, studies have reported hematological and renal toxicity in 17% of patients; pancytopenia in 5%; grade III or IV lymphoctyopenia in 23%, grade III anaemia in 3% and grade II renal insufficiency in 3% of patients (101-103), whereas no endocrine dysfunction of the pituitary axis (thyroid, adrenal, gonads) and no diabetes mellitus were seen (104). Interestingly, the novel DOTA-tagged SRIF-analog ^{177}Lu-octreotate showed grade III

anaemia, leucocytopenia and thrombocytopenia in 0%, 1% and 1% of administrations, respectively (83). Also, serum creatinin and creatinin clearance did not alter significantly, making this PRRT directed drug again suitable for clinical application in the near future. However, a 80% decrease in inhibin-B with a concurrent rise in FSH suggests a negative effect on spermatogenesis in men by [^{90}Y-DOTA^{0}, Tyr^{3}]octreotide and by [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate (82). Finally, the renal uptake of small radiopeptides, mediated through reabsorption in the proximal tubular cell of the kidney, can be reduced by the intravenous infusion of basic amino acids lysine and arginine, before, during and after the injection of the radioligand, thereby reducing renal toxicity (82, 105).

Conclusion

So far, only three SRIF-receptor ligands are approved for clinical use: SRIF itself and the metabolically stabilized octapeptide SRIF-analogs octreotide and lanreotide, both registered for medical treatment of acromegaly and GEP tumours. After their clinical introduction in 1987, the current limited number of proven indications for the use of SRIF-analogs in clinical medicine, has encouraged researchers for many years to unravel the (patho-) physiological role of SRIF throughout the human body. The identification of five distinct SRIF-receptor subtypes, being expressed on five different chromosomes, suggested different functions in different organs and provided specific targets for SRIF-analog chemistry and creating new therapeutic opportunities. In a very short time, not only sst subtype selective ligands and universal binding ligands have been discovered but also promising sst antagonists were introduced, whereby each group of compounds significantly participated to the current knowledge in the exciting field of SRIF-physiology. Subsequently, distinct, but overlapping patterns in function and the expression of these different sst subtypes in different (pathological) tissues have been demonstrated. Moreover, new observations on the physiology and interaction of various sst subtypes and other G-protein coupled receptors, i.e. homo-and heterooligomerization, might make the long-term use of new SRIF-ana-

logs with a different affinity profile in the treatment of (neuro-) endocrine tumours and cancer more successful. Also, sst-targeted radio- or chemotherapy of inoperable sst-positive cancer is an exciting new possibility, with promising early clinical observations. Still, the careful clinical evaluation of new SRIF-analogs (and antagonists) remains crucial, regarding their pitfalls, such as bioavailability, tachyphylaxis as well as potential adverse effects, since their novel pharmacological properties have usually been characterized in preclinical models only. Nevertheless, 30 years of tremendous efforts still provokes chemists, biologists and physicians to seek for better tools to fully understand the precise basic physiological role of SRIF and its receptors, thereby directly providing new medical treatment options for a variety of challenging diseases, including cancer.

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Chapter VI-2

Tachyphylaxis of somatostatin receptors is differentially regulated by somatostatin analogs

Abstract

It is well documented that in a subgroup of neuroendocrine tumors, the potent inhibitory responses to Octreotide (OCT) treatment diminish with continued exposure to this somatostatin (SRIF) analog. This phenomenon is know as adaptation or tachyphylaxis of inhibition of hormone secretion by SRIF-analogs. This report investigated the susceptibility of the SRIF receptor subtype (sst) 2 and sst_5, expressed either alone or together in HEK 293 cells, for tachyphylaxis to various SRIF-analogs. Pre-treatment during 24 h with 10 and 100 nM OCT resulted in a nearly total loss of responsiveness of the sst_2 receptor. The multiligand SOM230 (10 and 100 nM), induced a loss of responsiveness of sst_2 that can be classified as partial tachyphylaxis of SRIF-analog induced inhibition of adenylyl cyclase activity. Comparable partial tachyphylaxis was observed if sst_2-expressing cells were pre-treated with the sst_2-specific analog BIM-23120 (10 nM). In addition, sst_5 also seems less susceptible to tachyphylaxis compared with the sst_2 subtype, since SOM230 pre-treatment (10 and 100 nM) in sst_5-expressing cells resulted in a lower induction of tachyphylaxis compared with the (almost) complete tachyphylaxis induced by OCT in sst_2-expressing cells. Interestingly, no loss of sensitivity of sst_5 was found if sst_5-expressing cells were pretreated with OCT. Finally, the potencies and efficacies of SOM230 and OCT after pre-treatment with either OCT or SOM230 in the sst_2 + sst_5 co-transfected cells reflected the resultant of the SRIF-analog induced tachyphylaxis in the mono-transfected state. In conclusion, we demonstrate that the prolonged activity of agonists with very good binding affinity for sst_2 and/or sst_5 differentially elicit tachyphylaxis of both the sst_2 and the sst_5 receptor, presumably due to distinct agonist-induced receptor conformations.

Introduction

The biological actions of somatostatin (SRIF) are mediated via five G-protein coupled receptors (GPCR), named sst_1, sst_2, sst_3, sst_4 and sst_5. The high density of sst on human neuro-endocrine tumors originating from normal SRIF-target tissues has been used clinically to treat symptoms of hormonal hypersecretion in patients with growth hormone (GH)- or thyrotropin (TSH)-secreting pituitary adenomas, as well as in patients harbouring islet cell or carcinoids tumors, with SRIF-analogs (1). Moreover, the pres-

ence of sst_2 is a prerequisite for sensitivity of inhibition of tumour-related hormonal hypersecretion to treatment with octapeptide SRIF-analogs, i.e. Octreotide (OCT) and Lanreotide, as has been extensively demonstrated in patients with GH-secreting pituitary adenomas and islet cell tumours (2, 3). These currently clinically available octapeptides display a high membrane binding affinity for sst_2, a moderate affinity for sst_3 + sst_5 and no affinity for sst_4. However, in patients with islet cell or carcinoid tumors these initially potent inhibitory responses diminish with continued exposure (1, 4). Quite remarkably, this adaptation or tachyphylaxis of inhibition of hormone secretion by OCT and Lanreotide is not observed in patients with GH-secreting pituitary adenomas (5, 6). Continuous exposure to SRIF or SRIF-analogs may be associated with processes as receptor phosphorylation, G-protein uncoupling, receptor internalization, and degradation/down-regulation (7). As much of the current clinical knowledge with respect to tachyphylaxis is built upon sst_2-mediated mechanisms, however, possible novel therapeutical directions might become possible regarding targeting other sst subtypes, such as sst_1 and sst_5, since cumulating reports demonstrate their presence in neuro-endocrine tumors as well (5). The recently developed SRIF multiligand, SOM230 (8), seems of interest for the medical treatment of patients harbouring OCT-resistant neuroendocrine tumors. SOM230, in striking contrast to OCT, showed signs of only partial loss of its inhibitory effects on IGF-I levels during a period of 126 days continuous infusion in rats (9). The superior binding affinity of SOM230 for sst_5, compared with OCT, could well account for this enhanced IGF-I suppression (9). This would suggest, however, that sst_5 is not or less susceptible to tachyphylaxis by prolonged treatment with SRIF-analogs. In the present study, various SRIF-analogs, sst_2- or sst_5-specific, as well as universal, were studied in HEK 293 cells, transfected with sst_2, sst_5 alone or in combination, for their ability to induce tachyphylaxis of SRIF-analog mediated inhibition of forskolin (FSK)-induced cAMP release. Prolonged SRIF-analog treatment of sst-expressing HEK 293 cells, a model cell line that has been used extensively for studies of GPCR function, indicates that, there is ligand-specific induction of tachyphylaxis of the sst_2 and sst_5 receptor subtypes.

Methods

Cell line culture and sst constructs

HEK 293 cells (kind gift of Dr. A.P.N. Themmen, Internal Medicine, Erasmus MC Rotterdam, the Netherlands) were routinely passaged by trypsinization as described in detail previously (10). The cells were maintained in 75 cm^2 flasks in DMEM/F-12 medium, supplemented with non essential amino acids, sodium pyruvate (1 mmol/L), 10% fetal calf serum (FCS), penicillin ($1*10^5$ U/L), streptomycin (50 mg/L), fungizone (0.25 mg/L), L-glutamine (2 mmol/L), and sodium bicarbonate (2.2 g/L), pH 7.6. The cells were cultured at 37° C in a CO_2-incubator. Before transfection, the cells were seeded at 40% confluence in 75 cm^2 flasks and transfected the next day using the calcium phosphate precipitation method. In order to create different sst_2/sst_5 ratios being expressed, HEK 293 cells were transiently transfected with various concentrations of human sst_2 and sst_5 cDNA [human sst_2 or sst_5 cDNA in pBluescript (pBS) (a kind gift of G.I. Bell, Howard Hughes Medical Institute Chicago, Illinois) was excised from pBS and inserted into the Nhe-1/Sal1 or EcoRI/XbaI cloning site, respectively, of the mammalian expression vector pCi-neo (Promega Benelux, Leiden, the Netherlands)]. Media and supplements were obtained from GIBCO Bio-cult Europe (Invitrogen, Breda, the Netherlands).

Forskolin-induced cAMP Response Element-Luciferase Reporter Gene Assay

The functional responses of the SRIF-analogs in the various cell systems were determined using a cAMP-responsive reporter construct that contains six cAMP response elements in tandem in front of the cDNA encoding the luciferase (LUC) reporter enzyme [pCRE6lux (11)]. HEK293 cells were co-transfected with pCRE6Lux (11) and pRSVlacZ, to control for transfection efficiency (12) (maximum of 10 µg sst-expression construct, 2 µg pRSVlacZ, 2 µg pCRE6Lux, and 6 µg carrier DNA per ml precipitate). Two days after transfection, cells were pre-incubated for 24 h (at 37°C in culture medium) without or with SRIF-analog (10 or 100 nM). Subsequently,

media were refreshed and the SRIF-analog dependent CRE-LUC response was determined in 48-well tissue culture plates (Costar, Cambridge, MA) by incubating the cells for 6 h in culture medium containing 0.1% BSA with 1 µM forskolin (FSK) and increasing concentrations of SRIF-analog (range 100 nM – 0.01 pM). Thereafter, the media were aspirated, the cells lysed and luciferase activity was measured using a TopCount luminometer after adding luciferin (13). β-Galactosidase activity of the lysates was determined to correct for transfection efficiency (12). The CRE-LUC response produced by 1 µM FSK in each experiment was set at 100%.

Quantitative PCR

Quantitative PCR was performed as described previously (14). Messenger RNA was isolated using Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway) from transiently transfected (72 h) HEK 293 cells. The cells were lysed for 2 min in an ice-cold Tris-buffer (100 mM Tris-HCl, pH 8, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM DTT and 5 U/100µl RNAse inhibitor (HT Biotechnology Ltd., Cambridge, UK). The mixture was centrifuged at 14,000 rpm for 1 min to remove cell debris. After adding 40 µl pre-washed Dynabeads Oligo $(dT)_{25}$ to the supernatant, the mixture was incubated for 5 min on ice. Thereafter, the beads were collected with a magnet, washed three times with a Tris-buffer (10 mM Tris HCl, pH 8, 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS), and once with a similar buffer from which LiDS was omitted. Messenger RNA was eluted from the beads in 2 x 20 µl H_2O for 2 min at 65 °C. Complementary DNA (cDNA) was synthesized using the poly A^+ mRNA in a Tris-buffer (50 mMTris-HCl, pH 8.3, 100 mM KCl, 4 mM DTT, 10 mM $MgCl_2$) together with 1 mM of each deoxynucleotide triphosphate, 10 U RNAse inhibitor, and 2 U AMV Super Reverse Transcriptase (HT Biotechnology Ltd., Cambridge, UK) in a final volume of 40 µl. This mixture was incubated for 1 h at 42 °C. One tenth of the cDNA library was used for quantification of sst subtype mRNA levels. The assay was performed using 15 µl TaqMan Universal PCR master mix (Applied Biosystems, Capelle aan de IJssel, The Netherlands), 500 nM forward primer, 500 nM reverse primer,

100 nM probe and 10 µl cDNA template, in a total reaction volume of 25 µl. The reactions were carried out in a ABI 7700 sequence detector (The Perkin-Elmer Corporation, Groningen, The Netherlands). PCR amplification started with a first step for 2 min at 50 °C, followed by an initial heating at 95 °C for 10 min and, subsequently, samples were subjected to 40 cycles of denaturation at 95 °C for 15 sec and annealing for 1 min at 60 °C. To ascertain that no detectable genomic DNA was present in the poly A^+ mRNA preparation, since sst genes are intron-less, the cDNA reactions were also performed without reverse transcriptase. The detection of hypoxanthine-phosphoribosyl-transferase (hprt) mRNA served as a control and was used for normalization of the sst subtype mRNA levels. The primer sequences that were used have been described in detail previously (15).

Table 1. Binding selectivity of SRIF-analogs for the five sst subtypes.

Compound	Binding affinity (IC_{50}, nM)				
	sst_1	sst_2	sst_3	sst_4	sst_5
Octreotide	280	0.4	7.1	>1000	6.3
SOM230	9.3	1.0	1.5	>100	0.2
BIM-23206	>1000	166	1000	>1000	2.4
BIM-23120	>1000	0.34	412	>1000	213.5

Data are from radioligand binding assays to membranes from transfected CHO-K1 cells (8, 30) and African green monkey kidney cells (8) expressing the different human sst subtypes. Values are from IPSEN (Culler, M.D.), and from Lewis and coworkers (8).

Test-substances

Octreotide (OCT, Sandostatin®) and SOM230 were from Novartis Pharma A.G., (Basel, Switzerland). Somatostatin-14 was purchased from Sigma Chemical Co. (St. Louis, MO). BIM-23206, a sst_5-subtype specific

analog, and BIM-23120, a sst_2-specific analog, were synthesized by IPSEN (Massachusetts, USA). Sst binding affinities are depicted in Table I.

Statistical analysis

Calculation of IC_{50} values for inhibition of FSK-induced cAMP response was made using GraphPad Prism version 3.02 (San Diego, CA). The statistical significance of the difference between the mRNA expression levels after 10 nM SRIF-analog pre-treatment were determined by using one-way analysis of variance (ANOVA). When significant overall effects were obtained by this method, comparisons were made using Newman-Keuls multiple comparisons test. Data are reported as means ± SEM of the indicated n values, unless otherwise specified.

Results

cAMP response in sst_2 or sst_5 transfected HEK 293 cells

It has been demonstrated that the design of experiments involving the pre-treatment of cells with high concentrations of SRIF or SRIF-analogs followed by measurement of surface receptor numbers by ligand binding or functional responses, can have important consequences for interpreting the results. The concentration of agonist accumulating in the medium can be sufficient to re-activate the receptor and it has been shown that there is a dynamic cycling of both somatostatin agonist ligands and receptors between the cell surface and internal compartments both during agonist treatment and after surface-bound agonist has been removed, unless steps are taken to prevent the re-activation of receptors by recycled agonist (16). Therefore, we first evaluated the inhibitory effects of 10 nM OCT to inhibit FSK-induced cAMP (CRE-LUC) response in sst_2-expressing HEK 293 cells when pretreated overnight (24 hr) with 10 nM OCT. As depicted in figure 1, compared with control (a rapid removal of the medium followed by immediate incubation with 10 nM OCT in the presence of 1μM FSK at 37°C for 6 hr), washing the cells once in DMEM or washing the cells once in DMEM followed by a 10 min incubation at 37°C in HBSS pH 5 (to dissociate cell surface-bound ligand), did not alter the inhibitory

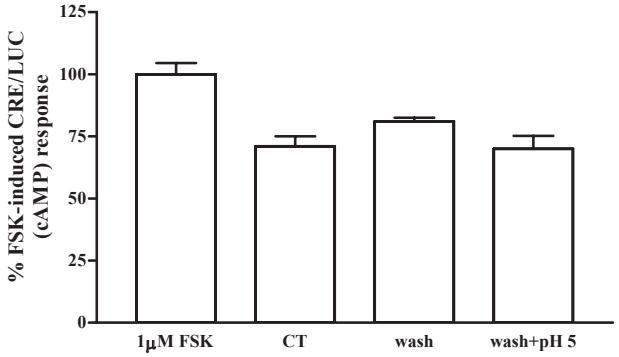

Figure 1. Control experiments in sst_2-expressing HEK 293 cells, to compare three commonly used strategies to handle cells that were pre-treated for 24 h with 10 nM OCT. cAMP response was determined with the use of six copies of a cAMP response element (CRE)-luciferase reporter gene construct (CRE-LUC). β-Galactosidase activity of the lysates was determined to correct for transfection efficiency. The cAMP (CRE-LUC) response produced by 1 μM FSK in each experiment was used as 100%. The values represent the means ± S.E. from separate experiments performed in quadruplicate. Each bar is representative for two individual experiments. CT, medium was removed from the wells and immediate incubation with FSK and 10 nM OCT in serum free DMEM was initiated; Wash, medium was removed from the wells, cells were washed once in DMEM (37°C) followed by incubation with FSK + OCT and Wash + pH 5, medium was removed from the wells, cells were washed once in DMEM (37°C) followed by 10 min incubation with HBSS (pH 5) at 37°C. Subsequently, HBSS was removed an incubation with FSK + OCT was initiated.

efficacy of 10 nM OCT to inhibit FSK-induced cAMP response (-29 ± 7, -19 ± 3 and -30 ± 9%, respectively). In addition, the average cAMP response produced by 1 μM FSK in the untreated experiments (5 ± 2 fold over control) was not different compared with SRIF-analog pretreated experiments (6.5 ± 2.5 fold over control). In subsequent experiments, therefore, after pre-treating the cells with a SRIF-analog, rapid removal of the medium was followed by immediate incubation with 1μM FSK in the presence of a 100 nM - 1 pM range of SRIF analog at 37°C for 6 hr.

To investigate the susceptibility of the sst_2 receptor to tachyphylaxis, sst_2-expressing HEK 293 cells were incubated in the absence or presence of three different SRIF-analogs (10 nM) for 20 h at 37°C. In untreated cells, OCT and SOM230 inhibited FSK-induced cAMP response with an IC_{50} of 0.02 ± 0.09 and 0.4 ± 0.23 nM, respectively. Maximum inhibition was 79 ± 1 and 90 ± 1%, respectively. Preincubation of cells with 10 nM OCT,

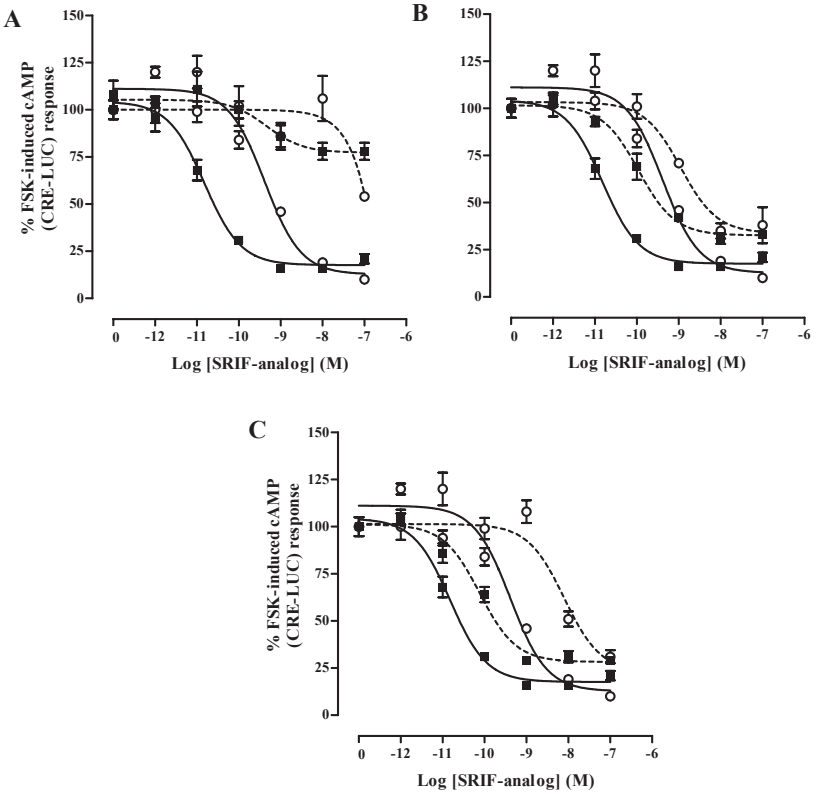

Figure 2. Dose-dependent inhibition of FSK-induced cAMP response by OCT (■) and SOM230 (○) in sst_2-expressing HEK 293 cells, that were untreated (solid lines) or pre-treated for 24 h (dashed lines) with 10 nM OCT (A), SOM230 (B) or BIM-23120 (C). cAMP response was determined with the use of six copies of a cAMP response element (CRE)-luciferase reporter gene construct (CRE-LUC). The values represent the means ± S.E. from separate experiments performed in quadruplicate. Each graph is representative for two individual experiments

as depicted in figure 2A, dramatically reduced the efficacy of maximum inhibition by OCT (-22 ± 5%) as well as the potency (IC_{50} = 0.5 ± 0.5 nM). In addition, the potency of SOM230 to inhibit the FSK-induced cAMP response was completely abolished after pre-treatment with 10 nM OCT (IC_{50} = > 1000 nM). Pre-treatment of cells with 10 nM SOM230 resulted in different responses. As can be seen in figure 2B, the potency of OCT was attenuated only 5-fold compared with untreated cells while the maximal inhibition was still -67 ± 1%. The same results were observed for SOM230; the potency lowered nearly 3-fold while the efficacy remained at -62 ± 9% suppression of FSK-induced cAMP response. Finally, sst_2-expressing

cells were pre-treated with 10 nM BIM-23120 (Fig. 2C), a sst_2-selective SRIF analog with comparable binding affinity *(*0.34 nM*)* and functional potency to suppress FSK-induced cAMP (0.03 ± 0.20 nM) response as OCT. Interestingly, treatment of cells with BIM-23120, increased the IC_{50} for OCT only 4-fold (0.08 ± 0.15 nM), while maximum inhibition remained -71 ± 1%. The IC_{50} of SOM230 after pre-treatment with 10 nM BIM-23120 appeared 7.9 ± 0.30 nM while its efficacy was -69 ± 2%. In untreated sst_5-expressing HEK 293 cells OCT and SOM230 inhibited FSK-induced cAMP response with an IC_{50} of 8.4 ± 0.10 and 0.05 ± 0.11 nM, respectively (Fig. 3). Maximum inhibition was 73 ± 31 and 82 ±1%,

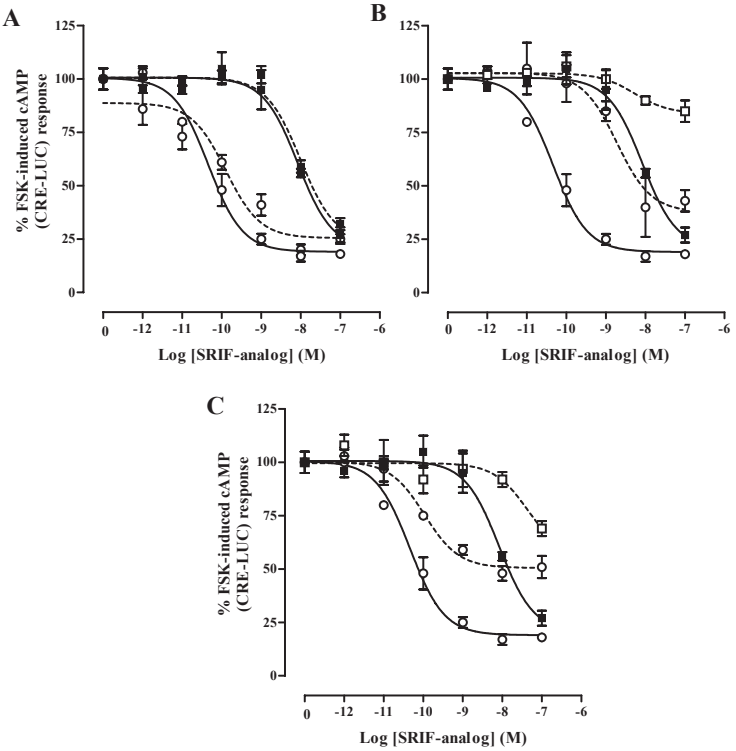

Figure 3. Dose-dependent inhibition of FSK-induced cAMP response by OCT (■) and SOM230 (○) in sst_5-expressing HEK 293 cells, that were untreated (solid lines) or pre-treated for 24 h (dashed lines) with 10 nM OCT (A), SOM230 (B) or BIM-23206 (C). cAMP response was determined with the use of six copies of a cAMP response element (CRE)-luciferase reporter gene construct (CRE-LUC). The values represent the means ± S.E. from separate experiments performed in quadruplicate. Each graph is representative for two individual experiments.

respectively. OCT pre-treatment did not affect both the potency as well as the efficacy of both SRIF-analogs to inhibit FSK-induced cAMP response (Fig. 3A: OCT, IC_{50} = 9.9 ± 0.20 nM and -68 ± 4%; SOM230, IC_{50} = 0.1 ± 0.30 nM and -75 ± 2%). The maximum inhibition by OCT was only 15 ± 5% when cells were pretreated with 10 nM SOM230. SOM230, on the other hand, suppressed FSK-induced cAMP response by 57 ± 5% while its potency was lowered to 1.9 ± 0.18 nM (Fig. 3B). Finally, sst_5-expressing cells were pretreated with 10 nM BIM-23206 (Fig. 3C), a sst_5-selective SRIF analog with high binding affinity (2.4 nM) and functional potency to suppress FSK-induced cAMP (IC_{50} = 0.20 ± 0.11 nM). Both the potency and efficacy of OCT were attenuated (45 ± 0.7 nM and -31 ± 4%) after pre-treatment with BIM-23206. The potency of SOM230, after BIM-23206 pre-treatment, was lowered 2-fold (0.1 ± 0.1 nM) while its maximum inhibition was partially reduced to -49 ± 6%.

mRNA expression in sst_2 or sst_5 transfected HEK 293 cells

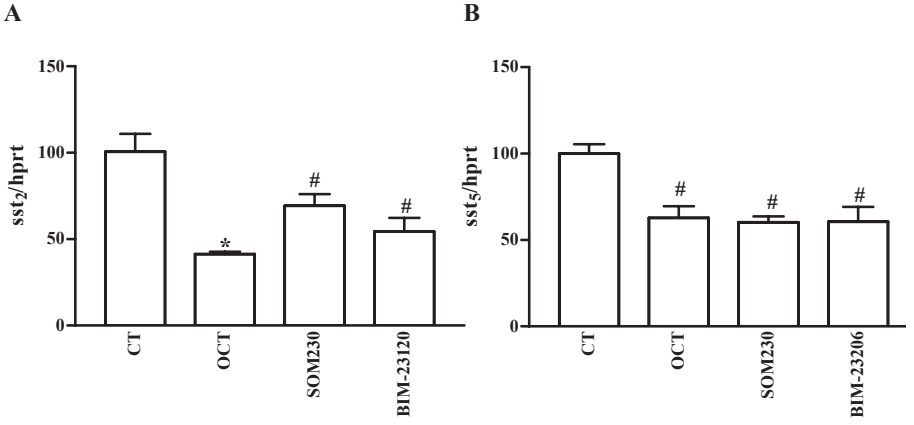

Figure 4. Expression of sst_2 (A) and sst_5 (B) mRNA levels in HEK 293 cells after 24h pre-treatment with various SRIF-analogs at 10 nM concentration. Sst and hprt mRNA levels were quantified by a TaqMan assay and results are depicted as bars, representing the means ± S.E. from 2 separate measurements performed in duplicate, and are adjusted for hprt expression. CT = control untreated cells. #, $p<0.05$ vs CT; *, $p<0.01$ vs CT.

As depicted in figure 4, all SRIF-analogs significantly suppressed sst_2 mRNA expression levels (Fig. 4A: range of inhibition 31-59%) as well as sst_5 mRNA expression levels (Fig. 4B: approximately 40% inhibition) when sst_2- or sst_5-expressing HEK 293 cells

were pretreated for 24 h with 10 nM of the individual compounds.

cAMP response in sst_2 + sst_5 co-transfected HEK 293 cells

Because a significant proportion of human sst-positive neuroendocrine tumors simultaneously express multiple sst subtypes, including sst_2 and sst_5 (5), we also evaluated tachyphylaxis for SRIF-analogs in sst_2 and sst_5 co-expressing HEK 293 cells. When the sst_2/sst_5 mRNA expression ratio was high (Fig. 5: sst_2, 2.87 ± 0.21 sst/hprt and sst_5, 0.34 ± 0.01 sst/hprt) in untreated HEK 293 cells,

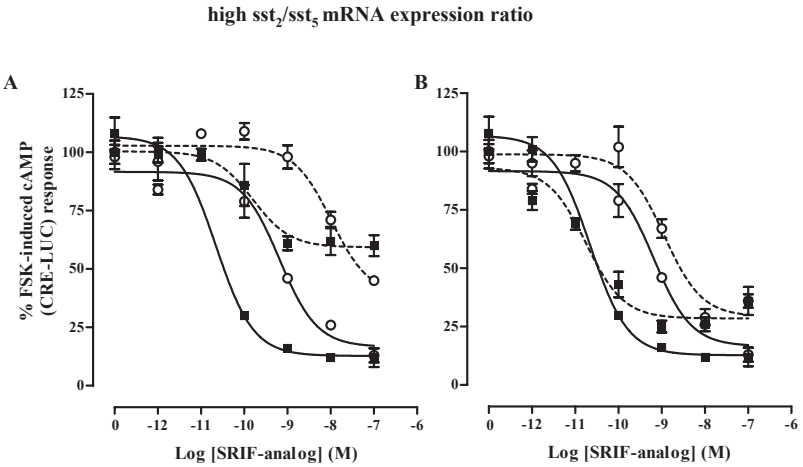

Figure 5. Dose-dependent inhibition of FSK-induced cAMP response by OCT (■) and SOM230 (○) in HEK 293 cells, with a high sst_2/sst_5 mRNA expression ratio, that were untreated (solid lines) or pre-treated for 24 h (dashed lines) with 10 nM OCT (A) or SOM230 (B). cAMP response was determined with the use of six copies of a cAMP response element (CRE)-luciferase reporter gene construct (CRE-LUC). The values represent the means ± S.E. from separate experiments performed in quadruplicate.

the potency in terms of IC_{50} for OCT and SOM230 was 0.01 ± 0.44 and 0.66 ± 0.17 nM, respectively. Maximum inhibition was -88 ± 4% for OCT and -87 ± 3% for SOM230. Preincubation of these cells with 10 nM OCT (Fig. 5A) attenuated the potency (IC_{50} = 0.16 ± 0.12 nM) and efficacy of OCT inhibition (maximum inhibition = -40 ± 5%) as well as the potency (IC_{50} = 10 ± 0.18 nM) and efficacy of SOM230 inhibition (maximum inhibition = -55 ± 2%). Treatment of cells with 10 nM SOM230, as depicted in figure 5B, only attenuated the potency of OCT

2 fold (IC_{50} = 0.02 ± 0.24 nM) and maximum inhibition remained -74 ± 4%. The same results were observed for SOM230: IC_{50} lowered nearly 2-fold to 1.14 ± 0.19 nM and maximum inhibition was -74 ± 6%. Subsequently, the same panel of experiments were carried out in HEK 293 cells, in which the sst_2/sst_5 ratio was low (Fig. 6: sst_2, 0.71 ± 0.09 sst/hprt and sst_5, 2.02 ± 0.20 sst/hprt). In untreated cells, the potency in terms of IC_{50}

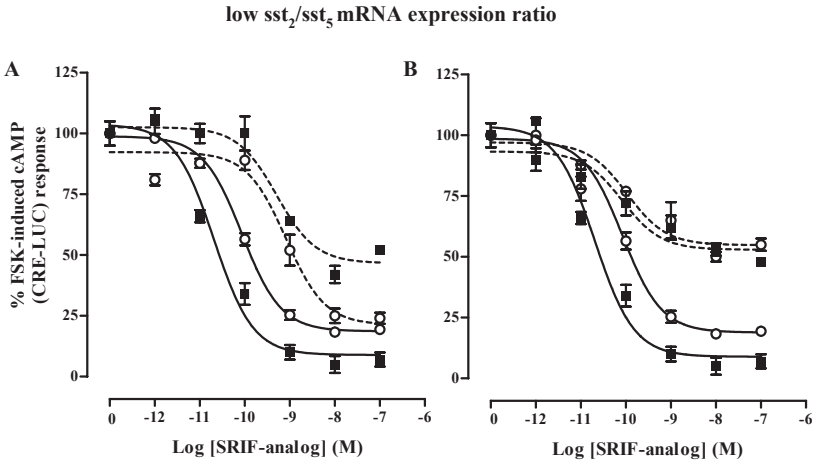

Figure 6. Dose-dependent inhibition of FSK-induced cAMP response by OCT (■) and SOM230 (○) in HEK 293 cells, with a low sst_2/sst_5 mRNA expression ratio, that were untreated (solid lines) or pre-treated for 24 h (dashed lines) with 10 nM OCT (A) or SOM230 (B). cAMP response was determined with the use of six copies of a cAMP response element (CRE)-luciferase reporter gene construct (CRE-LUC). The values represent the means ± S.E. from separate experiments performed in quadruplicate.

for OCT and SOM230 was 0.02 ± 0.16 and 0.08 ± 0.04 nM, respectively. Maximum inhibition was -93 ± 4% for OCT and -81 ± 1% for SOM230. If the cells were pretreated with 10 nM OCT (Fig. 6A), both the potency and efficacy of OCT to inhibit FSK-induced cAMP were attenuated (IC_{50} = 0.51± 0.20 nM, -48 ± 1%). The potency of SOM230 was only lowered 4-fold (IC_{50} = 0.83 ± 0.25 nM) while its maximum inhibition appeared -76 ± 2%. Pre-treatment of cells with 10 nM SOM230 (Fig. 6B), lowered the potency of OCT 4-fold (IC_{50} = 0.08 ± 0.30 nM) and maximum inhibition was attenuated to -52 ± 1%, while the potency of SOM230 was unaffected (IC_{50} = 0.10 ± 0.33 nM) and maximum inhibition was lowered to -45 ± 3%.

Pre-treatment of HEK 293 cells at 100 nM SRIF-analog concentration

Finally, we investigated the susceptibility of both individual sst subtypes to tachyphylaxis in the presence of 100 nM OCT or SOM230.

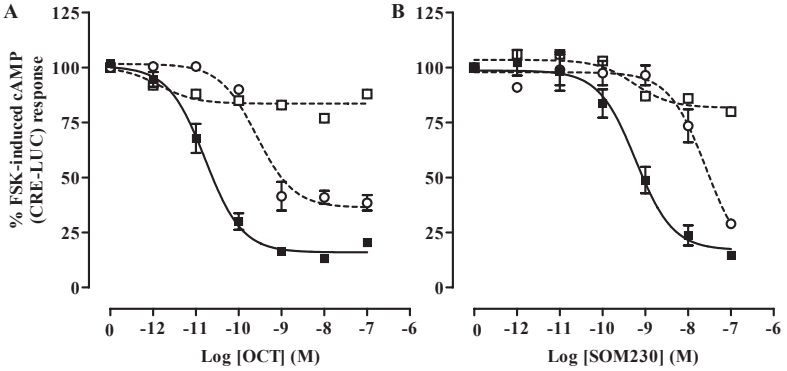

Figure 7. Dose-dependent inhibition of FSK-induced cAMP response by OCT (A) and SOM230 (B) in sst_2-expressing HEK 293 cells, that were untreated (■, solid line) or pre-treated (dotted lines) for 24 h with 100 nM OCT (□) or SOM230 (○). cAMP response was determined with the use of six copies of a cAMP response element (CRE)-luciferase reporter gene construct (CRE-LUC). The values represent the means ± S.E. from at least 2 separate experiments performed in quadruplicate.

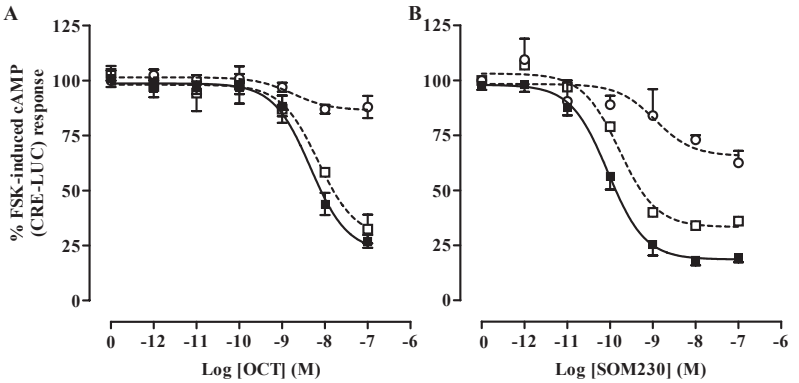

Figure 8. Dose-dependent inhibition of FSK-induced cAMP response by OCT (A) and SOM230 (B) in sst_5-expressing HEK 293 cells, that were untreated (■, solid line) or pre-treated (dotted lines) for 24 h with 100 nM OCT (□) or SOM230 (○). cAMP response was determined with the use of six copies of a cAMP response element (CRE)-luciferase reporter gene construct (CRE-LUC). The values represent the means ± S.E. from at least 2 separate experiments performed in quadruplicate.

Pre-treatment of sst_2-expressing cells with 100 nM OCT completely abolished the inhibitory effects of OCT and SOM230 on FSK-induced cAMP response (Fig. 7A and 7B). On the other hand, pre-treating the cells with 100 nM SOM230 lowered the potency of OCT only 10-fold: from 0.02 ± 0.08 nM to 0.26 ± 0.16 nM, while maximum inhibition went from -84 ± 2% in the absence to -62 ± 4% in the presence of 100 nM SOM230. Comparable effects were observed for SOM230: maximum inhibition was slightly reduced from -83 ± 4% to -69 ± 1% while the IC_{50} for SOM230 was increased from 0.60 ± 0.13 nM to 26 ± 0.16 nM. Strikingly, no significant alterations in terms of efficacy and potency for OCT and SOM230 to inhibit FSK-induced cAMP response by sst_5-expressing cells were observed, when pre-treated for 24 h with 100 nM OCT (Fig. 8A and 8B). However, pre-treatment with 100 nM SOM230 significantly diminished the inhibitory effects by OCT, while SOM230 still induced significant suppressive effects on FSK-induced cAMP response in sst_5-expressing cells: maximum inhibition was reduced from -82 ± 2% to -35 ± 6%, while the IC_{50} for SOM230 was increased from 0.08 ± 0.09 nM to 1.14 ± 0.50 nM.

Discussion

The clinical observation of tachyphylaxis to SRIF-analog therapy in the medical treatment of neuroendocrine tumors, such as carcinoids and insulinomas, has prompted physicians, biologists and chemists to develop novel therapeutic tools that can provide other treatment options in this subgroup of patients. Before such a pharmacological tool can be designed, however, fundamental insights regarding the mechanisms involved in tachyphylaxis to SRIF-analogs seem essential. This report evaluated the susceptibility of sst_2 and sst_5 subtypes, either expressed alone or together in HEK 293 cells, for tachyphylaxis to the currently clinically available sst_2-preferring analog OCT, as well as to the recently developed multiligand SOM230, which has superior binding affinity for sst_5. We demonstrate that tachyphylaxis is sst subtype specific, but moreover, different SRIF-analogs differentially induce tachyphylaxis of the same sst subtype.

To keep in close proximity with the *in vivo* physiological concentration of SRIF and therapeutic levels of OCT, which both are within the one-nanomolar range (1), we have chosen 10 nM as the pre-treatment SRIF-analog concentration in most experiments. Moreover, although we are aware of the fact that GPCR-function in terms of G-protein uncoupling and/or receptor internalization can be affected within minutes after activation, we were primarily interested in prolonged pre-treatment with SRIF-analogs because this is more representative for the clinical observations of SRIF-analog induced tachyphylaxis. In agreement with previous reports (16-18), pre-treatment with 10 nM OCT resulted in a nearly total loss of responsiveness of the sst_2 receptor. Interestingly, SOM230, with a very high binding affinity for sst_2, induced a loss of responsiveness of sst_2 that can be classified as partial tachyphylaxis. Moreover, even a 100 nM concentration of SOM230, compared with 100 nM OCT, was still not able to induce a complete tachyphylaxis of SRIF-analog induced inhibition of adenylyl cyclase activity in sst_2-expressing cells. Strikingly, a comparable partial tachyphylaxis was observed when sst_2-expressing cells were pre-treated with 10 nM BIM-23120, a sst_2-specific SRIF analog with a superior sst_2 binding affinity and functional potency that equals the sst_2-pharmacological properties of OCT. Two interesting conclusions can be deduced from these experiments: 1) although SOM230 binds with good affinity to sst_2, this multiligand cannot induce complete tachyphylaxis and 2) the differential agonist-induced tachyphylaxis, as shown with the three different SRIF-analogs, all having superior binding affinity and functional activity at the sst_2 subtype, seems in support for the concept of for distinct agonist-induced receptor conformations. In agreement with the latter, convincing data have recently demonstrated for the first time that the activity of SRIF-analogs to regulate receptor endocytosis and signaling are not tightly linked and compelling evidence for the induction of agonist specific states of the sst_2 receptor was provided as it was shown that sst_2-agonists differ in their potency ratios for inhibiting adenylyl cyclase and stimulating receptor internalization (19). Taken all this evidence together, a reasonable amount of proof is provided for the induction of agonist-specific conformations of the sst_2 receptor and it can

be suggested that no single measure of agonist activity is predictive of all post-receptor effects induced by agonists (19). Our observations with the sst_5 subtype, moreover, provide the first evidence that the same hypothesis can be transposed to this receptor as well. In addition, considering the equally superior binding affinity of SOM230 and OCT for the sst_5 and sst_2, respectively, the sst_5 also seems less susceptible to tachyphylaxis compared with the sst_2 subtype. SOM230 pre-treatment of sst_5-expressing cells resulted in only a partial induction of tachyphylaxis compared with the (almost) complete tachyphylaxis induced by OCT in sst_2-expressing cells. Even 100 nM SOM230 pre-treatment showed that SOM230 could still inhibit FSK-induced cAMP response in a dose-dependent manner in SOM230 pre-treated sst_5-expressing cells, while tachyphylaxis was complete when sst_2-expressing cells were pre-treated with 100 nM OCT. Another striking observation and, in a way, supporting the lower susceptibility for tachyphylxis, as well as the agonist-specific induction of tachyphylaxis of sst_5 may be derived from our observations that both 10 nM and 100 nM OCT pre-treatment in sst_5-expressing cells did not result in a significant loss of sensitivity of the cells for SRIF-analog induced inhibition of adenylyl cyclase activity. Finally, the potencies and efficacies of SOM230 and OCT after pre-treatment with either OCT or SOM230 in the sst_2 + sst_5 co-transfected cells reflect the resultant of the SRIF-analog induced tachyphylaxis in the mono-transfected state. In this respect, our data seem not supportive for hetero-dimerization by sst_2 and sst_5 receptor subtypes, as has been demonstrated for sst_2 and sst_3. It was shown that this newly formed dimer displayed enhanced functional properties such as a greater resistance of the sst_2-sst_3 dimer to SRIF-14 (1μM for 6 hr) induced desensitization compared with SRIF-14 induced desensitization of sst_2 homodimers (20). However, the authors only evaluated whether SRIF-14 treatment results in desensitisation of the sst_3 itself, when adenylyl cyclase inhibition was evaluated with a non-peptidyl agonist for sst_3. Since they also demonstrated that native SRIF-14 had a 10-30 fold greater potency for sst_3 compared with the non-peptidyl agonist, it could well be that SRIF-14, on the basis of ligand-induced receptor conformations, can still be effective in their co-transfected sst_2+sst_3 cells via sst_3 homodimers.

It should be emphasized that our results are not being discussed in terms of the "classical" densitization. This classical paradigm for signal transduction by GPCR states that the active form of the receptor stimulates heterotrimeric G proteins and, subsequently, this receptor is rapidly phosphorylated by G-protein-coupled receptor kinases (GRKs), which leads to β-arrestin recruitment followed by effective uncoupling of the GPCR from the G-protein (21). The receptor is thereby rapidly desensitized, and the signaling is stalled. Agonist binding has been shown to stimulate sst_2 receptor phosphorylation, is potentiated by overexpression of GRKs (18, 22, 23), and is correlated with receptor internalization (18, 24). Furthermore, β-arrestin recruitment to the sst_2 at the plasma membrane is stimulated by SRIF-14 (23, 25). Because a dominant-negative β-arrestin failed to inhibit sst_2 endocytosis (25), it was proposed that β-arrestin plays a role in desensitization rather than receptor internalization. In addition, Liu and coworkers recently demonstrated different interactions between sst_2 and a GFP-tagged β-arrestin-2 after SRIF-14 treatment or after treatment with the nonpeptide agonist L-779,976 (19). Although β-arrestin-2-GFP was also recruited to the plasma membrane sst_2 receptors after L-779,976 binding, the β-arrestin-receptor complex dissociated within minutes. This observation indicates that the SRIF-14 receptor complex binds β-arrestin with higher affinity than L-779,976-receptor complex. In addition, the sst_5 subtype was recently shown to exhibit a class A receptor-like trafficking pattern (23), *i.e.* a rapid recycling back to the plasma membrane after receptor activation by SRIF-14. Down-regulation of sst receptors may form another (long-term) cause of tachyphylaxis after continuous exposure of sst to agonists (5). Chronic exposure of cultured pituitary cells to relatively high concentrations of SRIF-14, SRIF-28, or SRIF-analogs reduces the number of sst on AtT20 and 7135b pituitary tumor cells (26-29). In both sst_2- and sst_5 mono-transfected cells we observed a lower quantitative mRNA expression level after prolonged SRIF-analog treatment. This supposed sst mRNA down-regulation was not agonist-specific, in contrast to the observed agonist-specific tachyphylaxis of sst_2 and sst_5. Furthermore, in another series of experiments it was shown that in cells expressing an amount of sst_2

or sst_5 mRNA that was comparable to the amount of sst_2 or sst_5 mRNA which was observed after SRIF-agonist treatment, this amount is still sufficiently high to produce concentration-dependent inhibition curves comparable to (untreated) control curves (unpublished data). Therefore, we suggest that sst down-regulation seems partially involved in tachyphylaxis, but other cellular mechanisms must be taken into account as well. What can be the clinical implications of our results? Prolonged OCT treatment in neuro-endocrine tumors, expressing both sst_2 and sst_5, may result in complete tachyphylaxis of the sst_2 subtype, but the sst_5 will remain functionally active. Therefore, it won't be of any use for subsequent treatment with a sst_2-selective or preferring SRIF-analog, but adjuvant treatment with SRIF-analogs that can bind with sub-nanomolar affinity to sst_5 might be of interest to elicit once again a therapeutic response in these OCT-resistant cases. On the other hand, prolonged treatment with SOM230 might result in a longer duration of action irrespective of the sst_2 and sst_5 expression levels, because SOM230 induces only partial tachyphylaxis of both receptor subtypes.

In conclusion, we demonstrate that agonists with a very good binding affinity for sst_2 and/or sst_5 differentially elicit tachyphylaxis of both the sst_2 and the sst_5 receptor. These results provide some new insights in the mechanisms involved in tachyphylaxis after prolonged SRIF-analog treatment and could be of help for the development of novel therapeutic tools for patients that are classified as OCT-resistant. Additional studies, however, are inevitable to determine which cellular mechanisms are actually triggered after the distinct agonist-induced receptor conformation.

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Chapter VI-3

Functional characterisation of the novel tri-selective chimeric molecule BIM-23A760 with activity at somatostatin receptors 2 and 5, and the dopamine D2 receptor

Abstract

The recent development of novel pharmacological agents targeting both somatostatin (SRIF) receptors and the Dopamine (DA) D2 receptor, *i.e.* chimeric SRIF-DA agonists, might lead to novel potential opportunities in the medical treatment of acromegaly. This report is the first that analysed BIM-23A760, a tri-chimeric molecule, both in a series of sst_2+sst_5+D2R transfected mammalian cells, to retrieve additional insights with respect to the functional explanation for the high potency of these chimeric molecules. While the membrane binding affinity of the tri-chimeric molecule BIM-23A760 compared with the internal control BIM-23023, for sst_2 and sst_5, display a 10-fold difference, respectively, no differences to inhibit forskolin (FSK)-induced cAMP response were observed in the sst_2 or sst_5 mono-transfected HEK 293 cells. Furthermore, while BIM-23A760 possesses a similar binding affinity for the D2R, compared with the D2R-targeted internal control BIM-53097, the chimera was even 20-fold more potent to dose-dependently suppress FSK-induced cAMP response. In various sets of co-expressing sst_2+sst_5+D2R HEK 293 cells the efficacy of BIM-23A760 in terms of maximal inhibitory effect (100nM) or concentration-dependent inhibition of FSK-induced cAMP response was not enhanced compared with the combination of BIM-23023 and BIM-53097 or Cabergoline. In conclusion, our results BIM-23A760 are not in favour of oligo-heterodimerization between sst_2, sst_5 and D2R, but merely suggest that dopastatin chimeras can alter ligand-mono-receptor complexes differently, that already can result in enhanced potency of these novel pharmacological agents.

Introduction

Recent evidence suggested potential cross-talk of somatostatin (SRIF) receptors with other members of the G-protein coupled receptor (GPCR) family. Heterodimerization of SRIF receptor subtype (sst) 5 and dopamine D2 receptors (D2R) seems to result in the formation of a novel receptor with possible enhanced biological activity (1). On the basis of these data, including the observation that combined SRIF-analog and dopamine (DA) agonist treatment inhibits growth hormone (GH) hypersecretion in a significant proportion of acromegalic patients partially resistant to Octreotide (OCT) or Lanreotide (2), Saveanu et al. (3) recently studied the

effects of the chimeric molecule BIM-23A387, which selectively binds with high affinity to sst_2 and D2R receptors, on GH and prolactin (PRL) release by 11 cultured GH-secreting pituitary adenomas. In both OCT-sensitive, as well as in cultures showing partial responsiveness to OCT, the maximal inhibition of GH release induced by the individual sst_2- and D2R analogs and by BIM-23A387 was comparable. However, the mean IC_{50} for GH suppression by BIM-23A387 (0.2pM) was 50 times lower than that of the individual sst_2 and D2R specific compounds. This enhanced potency of chimeric molecules, such as BIM-23A387, may therefore lead to potential novel medical treatment options in acromegalic patients. Recent insights show that sst_5 receptors are highly expressed in the majority of GH-secreting pituitary adenomas (4, 5). Therefore, in order to further extend the concept of SRIF/DA chimeras, recently a novel chimeric analog, BIM23A760, which in addition to sst_2 and D2R activity also has activity at the sst_5 receptor, has been developed (6). This report characterizes the novel BIM-23A760 molecule in a functional sst and D2R receptor assay, to sort out why chimeric molecules have enhanced potencies compared with the combined treatment of SRIF-analogs and Dopamine agonists.

Methods

Cell line culture and sst constructs

HEK 293 cells (kind gift of Dr. A.P.N. Themmen, Internal Medicine, Erasmus MC Rotterdam, the Netherlands) were routinely passaged by trypsinization as described in detail previously (7). The cells were maintained in 75 cm^2 flasks in DMEM/F-12 medium, supplemented with non essential amino acids, sodium pyruvate (1 mmol/L), 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 µg/ml), and fungizone (0.25 µg/ml), L-glutamine (2 mmol/L), and sodium bicarbonate (2.2 g/L), pH 7.6. The cells were cultured at 37° C in a CO_2-incubator. Before transfection, the cells were seeded at 40% confluence in 75 cm^2 flasks and transfected the next day using the calcium phosphate precipitation method. In order to create a series of different sst_2/sst_5/D2R ratios being expressed, HEK 293 cells were transiently transfected with various concentrations

of human sst_2, human sst_5 cDNA [human sst_2 or sst_5 cDNA in pBluescript (pBS) (a kind gift of G.I. Bell, Howard Hughes Medical Institute Chicago, Illinois) was excised from pBS and inserted into the Nhe-1/SalI and Eco-RI/XbaI, respectively, cloning site of the mammalian expression vector pCi-neo] and human D2R cDNA (commercially available at UMR cDNA resource center; www.cDNA.com). Media and supplements were obtained from GIBCO Bio-cult Europe (Invitrogen, Breda, the Netherlands).

Forskolin-stimulated cAMP Response Element-Luciferase Reporter Gene Assay

The functional responses of the SRIF-analogs in the various cell systems were determined using a cAMP-responsive reporter construct that contains six cAMP response elements in tandem in front of the cDNA encoding the luciferase (LUC) reporter enzyme [pCRE6lux (8)]. HEK293 cells were co-transfected with pCRE6Lux (8) and pRSVlacZ (9) (maximum of 10 µg sst-expression construct, 2 µg pRSVlacZ, 2 µg pCRE6Lux, and 6 µg carrier DNA per ml precipitate). Three days after transfection the SRIF-analog dependent cAMP (CRE-LUC) response was determined in 48-well tissue culture plates (Costar, Cambridge, MA) by incubating the cells for 6 h in culture medium containing 0.1% BSA with 1 µM forskolin (FSK) and increasing concentrations of SRIF-analog (range 10 µM – 0.01 pM). Subsequently, the media was aspirated, the cells lysed and luciferase activity was measured using a TopCount luminometer after adding luciferin (10). β-Galactosidase activity of the lysates was determined to correct for transfection efficiency (9). The CRE-LUC response produced by 1 µM FSK in each experiment was used as 100%.

Quantitative PCR

Quantitative PCR was performed as described previously (11). Messenger RNA was isolated using Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway) from transiently transfected (72 h) HEK 293 cells or freshly isolated pituitary adenoma cell pellets containing 1.0 X 10^6 adenoma cells. The cells were lysed for 2 min in an ice-cold Tris-buffer (100 mM

Tris-HCl, pH 8, 500 mM LiCl, 10 mM EDTA pH 8, 1% LiDS and 5 mM DTT. After adding 40 µl pre-washed Dynabeads Oligo $(dT)_{25}$ to the supernatant, the mixture was incubated for 10 min on ice. Thereafter, the beads were collected with a magnet, washed three times with a Tris-buffer (10 mM Tris HCl, pH 8, 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS), and once with a similar buffer from which LiDS was omitted. Messenger RNA was eluted from the beads in 2 x 20 µl H_2O for 2 min at 65 °C. Complementary DNA (cDNA) was synthesized using the poly A^+ mRNA in a Tris-buffer (50 mMTris-HCl, pH 8.3, 100 mM KCl, 4 mM DTT, 10 mM $MgCl_2$) together with 1 mM of each deoxynucleotide triphosphate, 10 U RNAse inhibitor, and 2 U AMV Super Reverse Transcriptase (HT Biotechnology Ltd., Cambridge, UK) in a final volume of 40 µl. This mixture was incubated for 1 h at 42 °C. One tenth of the cDNA library was used for quantification of sst subtype mRNA levels. The sst subtype assay was performed using 15 µl TaqMan Universal PCR master mix (Applied Biosystems, Capelle aan de IJssel, The Netherlands), 500 nM forward primer, 500 nM reverse primer, 100 nM probe and 10 µl cDNA template, in a total reaction volume of 25 µl. The forward primer, reverse primer and probe concentrations in the D2R assay were 300 nM, 300 nM and 200 nM, respectively. The reactions were carried out in a ABI 7700 sequence detector (The Perkin-Elmer Corporation, Foster City, CA). PCR amplification started with a first step for 2 min at 50 °C, followed by an initial heating at 95 °C for 10 min and, subsequently, samples were subjected to 40 cycles of denaturation at 95 °C for 15 sec and annealing for 1 min at 60 °C. To ascertain that no detectable genomic DNA was present in the poly A^+ mRNA preparation, since sst genes are intron-less, the cDNA reactions were also performed without reverse transcriptase. The detection of hypoxanthine-phosphoribosyl-transferase (hprt) mRNA served as a control and was used for normalization of the sst subtype mRNA levels. The primer sequences that were used have been described in detail previously (4). The D2R-primer sequences that were used were;
Forward: 5'- GCCACTCAGATGCTCGCC-3,
Reverse: 5'- ATGTGTGTGATGAAGAAGGGCA-3' and
Probe: 5 'FAM - TTGTTCTCGGCGTGTTCATCATCTGC-TAMRA-3

Test-substances

BIM-23A760, BIM-23023 and BIM-53097 were synthesized at IPSEN (Massachusetts, USA). Sst and D2R binding affinities are depicted in Table I.

Table I. Human sst and D2R binding affinities of the various DA and SRIF analogs. Values are from IPSEN.

Compound	sst_1	sst_2	sst_3	sst_4	sst_5	D2R
SRIF-14	1.95	0.25	1.2	1.8	1.4	ND
Octreotide	1140	0.6	34	7030	7	ND
Cabergoline	ND	ND	ND	ND	ND	3.0[a]
BIM-23023	6616	.42	87	2700	4.2	>1000
BIM-53097	ND	ND	ND	ND	ND	22.1
BIM-23A760	622	0.03	160	>1000	42	15.9

Values are the 50% inhibitory concentration (nM). ND, Not determined. a, Ref (31)

Statistical analysis

The statistical significance of the difference between the effects of 100-0.1 nM SRIF-analog in the CRE-LUC Reporter Gene Assay was determined by using ANOVA. When significant overall effects were obtained by this method, comparisons were made using Newman-Keuls multiple comparisons test. Calculation of IC_{50} values for inhibition FRSK-induced cAMP accumulation and inhibition of hormone release were made using GraphPad Prism version 3.02 (San Diego, CA). The unpaired Student t-test was chosen to analyze differences in concentration-effect curves. Data are reported as means ± SEM of the indicated n values, unless otherwise specified.

Results

cAMP Response

The concentration-dependent inhibition of FSK-induced cAMP response in sst_2-expressing HEK 293 cells, in terms of IC_{50}, by BIM-23A760 was comparable to its internal control BIM-23023 (0.04 ± 0.02 vs 0.02 ± 0.01 nM, respectively). The maximal inhibitory effect, induced by 100 nM of the individual compound, was not statistically different as well (Fig. 1A). Corresponding IC_{50} values in sst_5-expressing HEK 293 cells

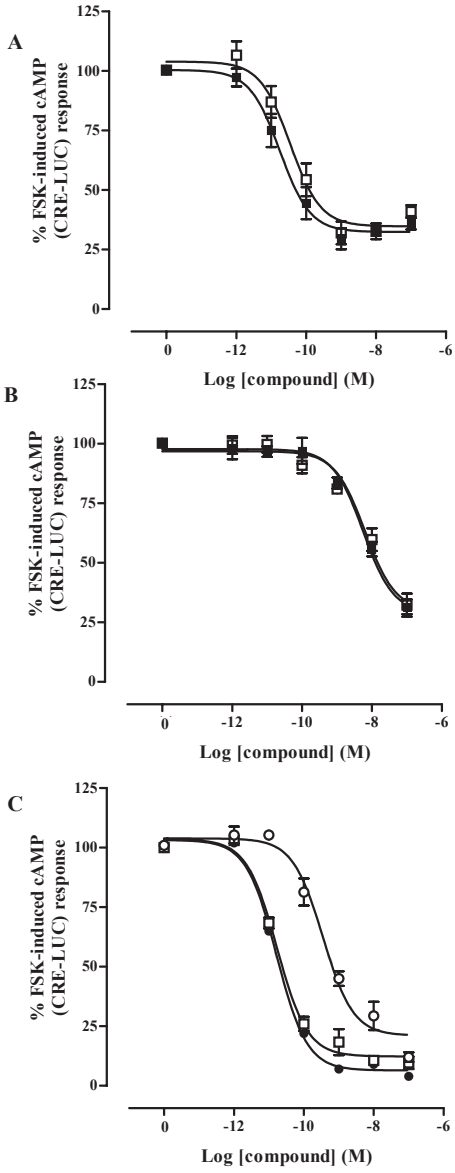

Figure 1. Dose-dependent inhibition of FSK-induced cAMP response by BIM-23023 (■), BIM-23A760 (□), BIM-53097 (○) and Cabergoline (●) in transiently transfected HEK 293 cells, expressing only sst_2 (A), sst_5 (B) or D2R (C). cAMP response was determined with the use of six copies of a cAMP response element (CRE)-LUC reporter gene construct. β-Galactosidase activity of the lysates was determined to correct for transfection efficiency. The CRE-LUC response produced by 1 μM FSK in each experiment was used as 100%. The values represent the means ± S.E. from at least 3 separate experiments performed in quadruplicate.

for the concentration dependent inhibition of FSK-induced cAMP response by BIM-23A760 and BIM-23023 were 6.3 ± 0.1 and 5.6 ± 0.1 nM, respectively (p=ns), while the inhibitory effects of 100 nM of the compounds were not statistically different (Fig. 1B). No statistical difference at 100nM concentration was observed between BIM-53097 and BIM-23A760 in D2-expressing HEK-293 cells as well (Fig. 1C).

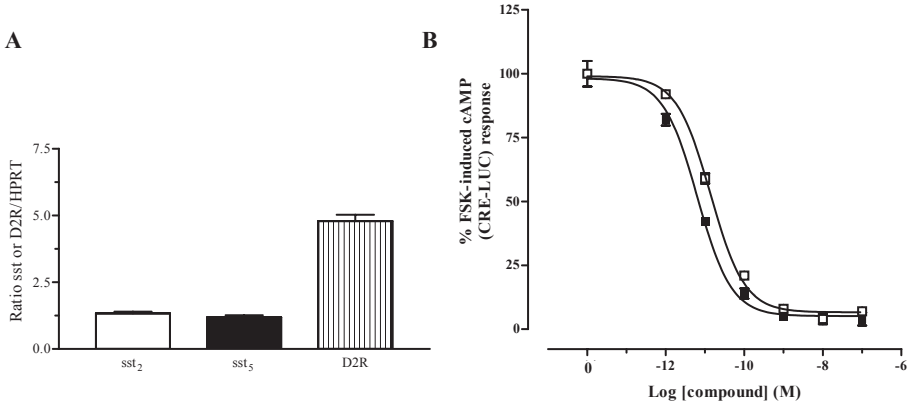

Figure 2. Functional characterisation of BIM-23A760 in transiently co-transfected HEK 293 cells, that expressed high D2R mRNA levels compared with sst_2 and sst_5. (A) Sst and D2R mRNA expression levels were quantified by a TaqMan assay and results are depicted as bars, representing the means ± S.E. from a single experiment performed in duplicate, and are adjusted for HPRT expression. sst_2 (open bars), sst_5 (black bars) and D2R (hatched bars) (B) Dose-dependent inhibition of FSK-induced cAMP response by BIM23A760 and BIM-23023+BIM52097. cAMP response was determined with the use of the CRE-LUC reporter gene construct. The values represent the means ± S.E. from a single experiment performed in quadruplicate. ■, BIM-23023+BIM-53097; □, BIM-23A760.

However, the concentration-dependent inhibition of FSK-induced cAMP response in D2R-expressing HEK 293 cells by BIM-23A760, IC_{50} value of 0.02 ± 0.07 nM, shifted 20-fold to the left, indicating increased potency, compared with its internal control BIM-53097 (IC_{50}: 0.4 ± 0.11 nM, $p<0.001$ vs. BIM-23A760). Cabergoline, the D2R agonist, displayed comparable efficacy as compared to BIM-23A760 in D2R-expressing HEK 293 cells (Fig. 1C, IC_{50}: 0.02 ± 0.07 nM). Subsequently, three sets of co-expressing sst_2+sst_5+D2R HEK 293 cells were synthesized and the efficacy of BIM-23A760 was compared with the combination of BIM-23023 and BIM-53097 or Cabergoline. No statistically differences in the maximal inhibitory effect (100nM) or

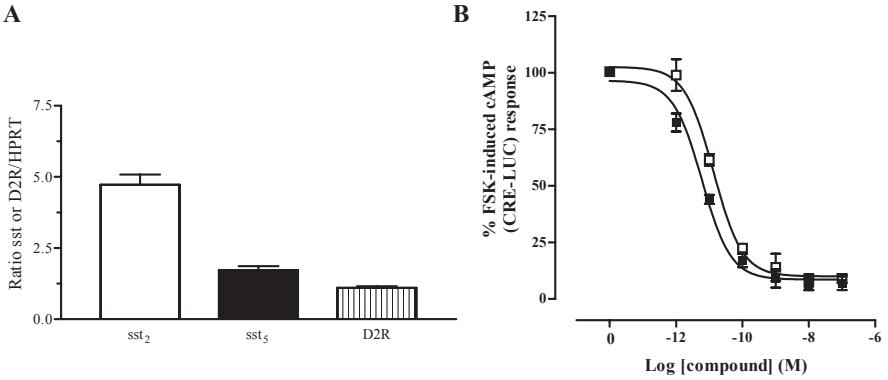

Figure 3. Functional characterisation of BIM-23A760 in transiently co-transfected HEK 293 cells, that expressed high sst_2 mRNA levels compared with sst_5 and D2R. (A) Sst and D2R mRNA expression levels were quantified by a TaqMan assay and results are depicted as bars, representing the means ± S.E. from a single experiment performed in duplicate, and are adjusted for HPRT expression. sst_2 (open bars), sst_5 (black bars) and D2R (hatched bars) (B) Dose-dependent inhibition of FSK-induced cAMP response by BIM23A760 and BIM-23023+Cabergoline. cAMP response was determined with the use of the CRE-LUC reporter gene construct. The values represent the means ± S.E. from a single experiment performed in quadruplicate. ■, BIM-23023+Cabergoline; □, BIM-23A760.

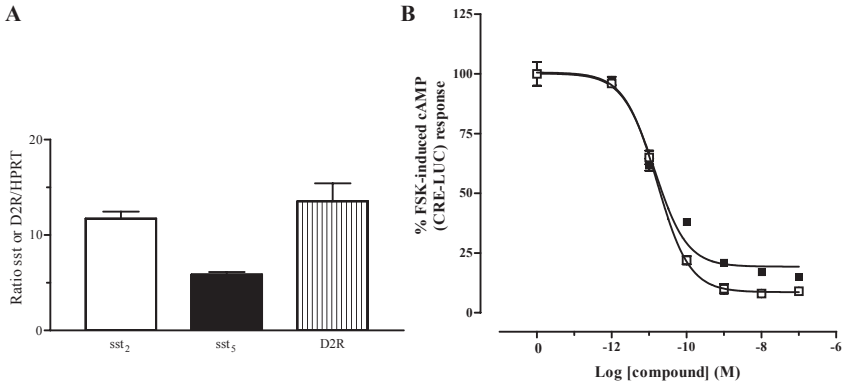

Figure 4. Functional characterisation of BIM-23A760 in transiently co-transfected HEK 293 cells, that expressed comparable mRNA levels of sst_2 and D2R and lower sst_5 mRNA expression. (A) Sst and D2R mRNA expression levels were quantified by a TaqMan assay and results are depicted as bars, representing the means ± S.E. from a single experiment performed in duplicate, and are adjusted for HPRT expression. sst_2 (open bars), sst_5 (black bars) and D2R (hatched bars) (B) Dose-dependent inhibition of FSK-induced cAMP response by BIM23A760 and BIM-23023+Cabergoline. cAMP response was determined with the use of the CRE-LUC reporter gene construct. The values represent the means ± S.E. from a single experiment performed in quadruplicate. ■, BIM-23023+Cabergoline; □, BIM-23A760.

concentration-dependent inhibition of FSK-induced cAMP response, in terms of IC_{50}, was observed between treatments, neither when D2R mRNA expression was higher than sst_2 or sst_5 expression (Fig. 2), nor when sst_2 mRNA expression was higher compared with sst_5 and D2R Fig. 3). As shown in Figure 4, when sst_2 equalled D2R mRNA expression, IC_{50} values were comparable between BIM-23A760 and BIM-23023+Cabergoline (0.02 ± 0.01 vs. 0.01 ± 0.1 nM, respectively).

Discussion

Although the clinical introduction of SRIF-analogs has nowadays emerged into their use as reference drug for the medical treatment of acromegaly, up to date, no more than approximately two thirds of cases of persistent acromegaly respond satisfactory to these agents (12). The recent development of novel pharmacological agents targeting both sst and D2R, *i.e.* chimeric SRIF-DA agonists, might lead to novel potential opportunities in the medical treatment of acromegaly. While the increased potency of a sst_2/D2R-interacting chimeric molecule, BIM-23A387, in suppressing GH and PRL secretion from human pituitary adenoma cells in vitro has been independently reported by two groups (3, 13), the underlying mechanism for this enhanced potency is not known yet. Nevertheless, a next generation of chimeric compounds has already been developed. Since cumulating evidence demonstrates high levels of sst_5 expression in GH-secreting pituitary adenomas (4, 5, 14-16) that might be of interest to suppress GH release, in particular if sst_2-targeted SRIF-analogs (OCT and Lanreotide) are (partially) ineffective (5, 17-19), the newest chimeric compounds display activity at sst_2+sst_5 and the dopamine D2 receptor. This report is the first that analysed BIM-23A760, a tri-chimeric molecule, to retrieve additional insights with respect to the functional explanation for the potency of these chimeric molecules. On the basis of the sst membrane binding affinity profile of the tri-chimeric molecule BIM-23A760 and its internal control BIM-23023, for sst_2 and sst_5, discrepancies with the functional (CRE-LUC) cAMP responses were observed in the mono-transfected HEK 293 cells, expressing only

sst_2 or sst_5. Although BIM-23A760, compared with BIM-23023, has a 10-fold higher binding affinity for sst_2 and a 10-fold lower binding affinity for sst_5, no differences were observed in concentration-dependent inhibition as well as in maximum suppression of FSK-induced cAMP accumulation. Moreover, while BIM-23A760 possesses a comparable binding affinity for the D2R, compared with the D2R-targeted internal control BIM-53097, the tri-chimeric molecule was 20-fold more potent to suppress FSK-induced cAMP response in a dose-dependent manner in the functional CRE-LUC assay. In addition, in the D2R mono-transfectant Cabergoline and BIM-23A760 showed comparable potency, despite the fact that Cabergoline has a five-fold higher binding affinity for the D2R (Table1). On the basis of this discrepancy between receptor binding and receptor activation seems interesting to speculate whether this enhanced functional potency of the mono D2R by BIM-23A760 might play a role in the demonstrated enhanced potency of chimeric compounds in vitro (3, 13). Therefore, it could be hypothesized that BIM-23A760 alters the mono ligand-receptor complex differently compared with individual DA or SRIF agonists. For example, receptor trafficking may be influenced differently resulting in enhancement of the receptor in terms of adenylyl cyclase inhibition. Recently, ligand specific-receptor trafficking by various SRIF-agonists for the sst_2 has been nicely demonstrated (20). It was shown that both cAMP inhibition and endocytosis were affected differently between sst_2-binding SRIF-analogs. These data seem to correspond with our data and, together, support the concept that ligand-receptor complexes within the G-protein coupled receptor family seem to be uniquely triggered by each individual ligand. As already shortly outlined above, the hybrid 'dopastatin' molecule BIM-23A387 induced in both OCT-sensitive, as well as in cultures showing partial responsiveness to OCT, a comparable maximal inhibition of GH release compared with the individual sst_2 and D2DR analogs. On the other hand, the mean IC_{50} for GH suppression by BIM-23A387 (0.2pM) was 50 times lower than that of the individual sst_2 and D2DR specific compounds. Recently, in a series of primary GH-secreting pituitary adenoma cultures collected from acromegalic patients classified as only

partially responsive to OCT, the tri-chimeric molecule BIM-23A760 was also demonstrated to have increased efficacy to suppress GH release by the adenoma cells, compared with the individual sst_{2+5} and D2R agonist (21). In 6 primary cultures, compared with OCT, BIM-23A760 increased maximal inhibition of GH release by 15%. Also, BIM-23A760 produced greater maximal suppression (± 13%) of GH secretion than the combination of the internal controls for sst_{2+5} and D2R, a finding that has not been reported for BIM-23A387. The higher efficacy of the chimeric compound can be explained, at least in part, by its higher binding affinity for sst_2. Quite surprisingly, no differences in efficacy between BIM-23A760 and the combination of BIM-23023 and BIM-53097 or Cabergoline, in terms of maximal suppression and dose-dependent inhibition of FSK-induced cAMP response were found when all three receptors where co-transfected at different expression levels in HEK 293 cells. Still, the in vitro data with BIM-23A387 and the use of sst_2 and D2R antagonists do suggest that sst and D2R can form oligo-heterodimers with distinct functional properties (3, 13). Ligand-induced oligomerization of G-protein coupled receptors has now been demonstrated for different receptors. Both sst_2 and sst_3 homodimers underwent agonist induced endocytosis, but the heterodimer of sst_{2a} and sst_3, both being over-expressed in HEK293 cells, dissociated at the cell membrane and only sst_2 underwent agonist induced endocytosis combined with loss of sst_3 function (22). In addition to this communication between receptor subtypes within the same GPCR family, sst_{2A} receptor heterodimerization with the μ-opioid receptor (MOR1) has also been demonstrated (23). The sst_{2A}-MOR1 heterodimers did not significantly alter the ligand binding or coupling properties but promoted cross-modulation of phosphorylation, internalization and desensitization of these receptors. Finally, studies using bioluminescence resonance energy transfer assays (BRET) on living cells have also indicated that hetero-oligomerization of the sst_5 and the D2R occurs following to agonist binding (1). The "new receptor" formed by the heterooligomerization of the D2 receptor and sst_5 appeared pharmacologically distinct from its receptor homodimers, as it was characterized by a much greater affinity for binding both dopamine and SS-agonists,

directly associated with enhanced G-protein and effector coupling to adenylyl cyclase. Recently, the first data on heterodimerizaton *in vivo* has been demonstrated. In normal rat astrocytes, sst_2 and D_2 receptors formed hetero-oligomeric complexes, displaying properties distinct from the native receptors (24). Other examples of GPCR heterodimer-formation with increase in biological functions with adrenergic (25), opioid (26, 27) and γ-aminobutyric acid (28, 29) receptors have been reported as well. Our data with BIM-23A760 are not in favour of oligo-heterodimerization between sst_2, sst_5 and D2R, but additional experiments such as the use of sst- and D2R-antagonists or confocal microscopy, to confirm this will have to be performed. Cumulating *in vitro* data are present which suggest that heterodimerization leads to a distinct receptor with altered functional properties does not occur. We propose, in addition to the current knowledge about GPCR function, that dopastatin chimeras can alter ligand-mono-receptor complexes differently, resulting in enhanced potency of these novel pharmacological agents. Additional support for this hypothesis is formed by Jaquet and coworkers. When they treated primary GH-secreting pituitary adenoma cells for 30 minutes with BIM-23A761, another tri-chimeric dopastatin molecule, a rapid GH-suppressive effect that lasted 24h was found. If the adenoma cells were co-treated with BIM-23023 and BIM-53097, however, a slower GH suppression that was lost already at 12 h was observed (30). In addition, incubation periods used in several reports with the dopastatin molecules that demonstrated enhanced potency, were 12 and 20 hr (3, 13). In conclusion, novel chimeric dopastatin molecules can become of interest in the medical treatment of GH-secreting pituitary adenomas. Ideally, these molecules should have superior binding-affinity towards the sst_2, sst_5 and D2R, thus initiating a super-agonist ligand-receptor conformation for each individual receptor. Eventually, such a hybrid compound forms the ideal pharmacological tool to treat GH hypersecretion, independent of the varying sst and D2R expression levels on the pituitary adenomas.

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Chapter VII

General Discussion

The patho-physiological role of SRIF receptor subtypes (sst) in neuro-endocrine diseases has gained enhanced scientific interest in the past few years. The development of novel, promising SRIF-analogs, both sst-specific and universal ligands, seem promising as a tool to further increase fundamental insights in sst function. Eventually, this research should result in novel medical therapeutic opportunities in patients suffering from neuro-endocrine diseases. In the present thesis we have evaluated the functional role of sst in pituitary adenomas, not only from a clinical point of view, but also studies were conducted to obtain more insights in distinct functional properties of sst in neuroendocrine tumor cells.

Clinical and functional characterisation of SRIF receptor subtypes in Acromegaly

Acromegaly is a debilitating disease which is predominantly caused by a GH-secreting pituitary adenoma. Following upon the successful clinical introduction of octreotide (OCT) and Lanreotide, two cyclic SRIF-analogs with comparable characteristics, these stable octapeptides have become a mainstay of medical therapy for acromegaly. Twenty years of clinical practice show, however, that these clinically available SRIF-analogs achieve clinical and biochemical control in approximately two-third of acromegalic patients, both as primary or secondary therapy. The characterisation of the five SRIF receptor subtypes (sst) between 1993 and 1995 has shed a new light on the pharmacological basis behind the efficacy of OCT and Lanreotide. All five sst bind natural SRIF-14 with a comparable high affinity, but there are major differences in the binding affinities of OCT and Lanreotide. These octapeptides bind with a high affinity to sst_2, with a moderate affinity to sst_3 and sst_5, but do not bind to sst_1 and sst_4. The expression of a high density of sst on the adenoma cells, mainly sst_2, forms the basis for successful treatment of acromegalic patients with sst_2-preferring SRIF-analogs such as OCT and Lanreotide. The molecular rationale for the clinical experience that one-third of patients with acromegaly is not adequately controlled by treatment with octapeptide SRIF-analogs is probably formed by a variable expression of the five sst

in the adenomas of these patients. Indeed, OCT-sensitive GH-secreting pituitary adenomas seem to present with a high level of both sst_2 and sst_5 mRNA expression, two subtypes predominantly involved in the regulation of GH release by human fetal pituitary cells, as well as by GH-secreting pituitary adenomas. On the other hand, tumors which are partially sensitive to OCT, sst_2 mRNA expression seems lower, while sst_5 mRNA expression is significantly higher compared with the OCT-sensitive tumors. As described in **chapter III-1**, we also found a variable expression of sst mRNAs, predominantly sst_2 and sst_5, in our series of GH-secreting pituitary adenomas. Only selected cases expressed sst_1 and sst_3 mRNAs, suggesting that these sst subtypes are probably of less importance in this type of pituitary adenoma. Moreover, in most adenomas, sst_5 expression levels were higher, compared with sst_2 mRNA levels. Interestingly, however, a positive correlation was found between sst_2, but not sst_5 mRNA levels in the adenoma cells and the inhibitory potency of OCT on GH release *in vivo* and *in vitro*, as well as the effects of SOM230 and SRIF-14 *in vitro*. These data demonstrate that the sst_2 subtype is clearly a predominant receptor determining responsiveness to SRIF-analogs in acromegalic patients *in vitro*. The advantage of the multiligand SOM230 which binds to 4 of the 5 human sst, was clearly demonstrated in one OCT-resistant culture. In this particular case, expressing low sst_2 and high sst_5, SOM230 inhibited GH release with comparable efficacy as SRIF-14, thereby confirming the importance of the sst_5 receptor subtype in mediating GH release, when sst_2 levels are low. Moreover, compared with OCT, SOM230 is significantly more potent in suppressing PRL release by mixed GH/PRL-secreting adenoma and prolactinoma cells, which appeared to be related to the expression level of sst_5, but not sst_2. However, the potential clinical importance of sst_5 receptors in prolactinomas should be considered in view of the very high proportion of patients with prolactinomas that respond to DA agonist treatment with a normalization of PRL levels and tumor shrinkage. We concluded that SOM230 has a broad profile of inhibition of tumoral pituitary hormone release in the low nanomolar range, probably mediated via both sst_2 and sst_5 receptors. The higher number of responders of GH secreting pituitary adenoma cultures to SOM230, com-

pared with OCT, suggest that SOM230 has the potency to increase the number of acromegalic patients which can be biochemically controlled.

A first proof-of-concept trial with SOM230 in patients with active acromegaly was performed in our center to further analyse to clinical potential of this novel multiligand SRIF-analog. A single s.c. injection of 250 µg SOM230 was compared with 100 µg OCT, thereby compensating for the 2.5 lower binding affinity for sst_2. This study showed a comparable efficacy in suppressing circulating GH concentrations in 8 patients, and showed a significant enhanced GH suppression in 3 of 12 acromegalic patients, as described in **chapter III-2**. The observation that SOM230 demonstrated potent inhibitory effects upon GH release in patients with GH-secreting pituitary adenomas expressing a low amount of sst_2 and a high amount of sst_5, suggests that the sst_5 subtype may indeed play an essential role in mediating *in vivo* GH suppressive actions by SOM230 in OCT-partial responders. The enhanced efficacy of SOM230 can be explained by high sst_5 expression in this type of adenomas and its 40-fold higher binding affinity for sst_5 as compared with OCT. *In vivo* and *in vitro* data from our trial emphasize that the inhibitory effects on GH release by SRIF and its analogs are primarily mediated via sst_2, as seen in the group of 8 equal responders to OCT and SOM230. However, when sst_2 mRNA over sst_5 mRNA levels are low as demonstrated in one patient, a suppressive action upon GH concentrations via sst_5 receptors becomes evident as well.

Additional evaluation of the effects of SOM230 on the metabolism of acromegalic patients has been discussed in **chapter III-3**. Interestingly, single dose administrations of 100 and 250µg SOM230 persistently inhibited free IGF-I levels significantly after 48 h whereas OCT was only effective for 24 h. Free IGF-I seems seem more important in the temporal short-term feedback between GH secretion and the circulating IGF-I system in normal subjects. The inhibitory effects on free IGF-I could be explained by intracellular dynamics of sst_2 and sst_5 at the central level of the pituitary, combined with the 40-fold higher sst_5-binding affinity and the longer half life of SOM230 compared with OCT. SOM230 might

induce this sustained inhibition of free IGF-I levels via sst_2 as well because of its long half life as compared with OCT. Moreover, only in the subgroup of those 8 acromegalic patients that responded equally to OCT and 250μg SOM230 with respect to circulating GH concentrations, total IGF-I concentrations were lowered to a small and similar extent 24h after s.c. injection with 100μg OCT and 250μg SOM230. This suggests a GH-dependent inhibition of total IGF-I, as no effects on total IGF-I levels after single doses of SOM230 and OCT were observed in the total series of 12 acromegalic patients. In addition, SRIF-analogs can dose-dependently inhibit GH-induced IGF-I production by rat hepatocytes and perfused rat livers, expressing sst_2 and sst_3. However, it is not known yet whether this peripheral regulation of IGF-I levels is functional in the human liver as well. It remains unclear why differences in inhibitory effects of free and total IGF-I concentrations between the two drug treatments were observed. SRIF analogs also directly regulate IGFBP-1 levels, which can block IGF-1 bioavailability. Even though 100 and 250μg SOM230 administration significantly and dose-dependently increased IGFBP-1 levels, OCT treatment was more potent. The absence of a statistically significant correlation between GH or insulin and IGFBP-1 levels after both OCT and SOM230 administration might support a direct role for SRIF-analog induced release of IGFBP-1 in acromegaly, and, considering the superior action of OCT compared with SOM230 in stimulating IGFBP-1 levels in acromegaly, these data suggest a modulatory role of sst_2 in the direct regulation of IGFBP-1 levels by SRIF-analogs in acromegaly.

In patients with acromegaly, the effects of GH excess on insulin sensitivity are reflected by an increased incidence of impaired glucose tolerance, and even overt diabetes in untreated patients. Since the human pancreas expresses multiple sst on α- and β-cells, a deterioration of glucose homeostasis was expected during SRIF-analog treatment. The inhibitory effects of OCT on insulin secretion are transient, however. In general, the effects of octapeptide SRIF-analogs on glucose homeostasis appeared to be minor and clinically important effects on carbohydrate metabolism during long-term therapy in acromegaly are not routinely observed. Com-

pared with OCT, we observed comparable elevations of glucose concentrations during lunch after SOM230 administration (**chapter III-3**). On the other hand, 250µg SOM230 elicited an acute rise in glucose levels already 1 h post-injection. SOM230-mediated inhibitory effects on insulin secretion cannot explain these elevated glucose levels. On the basis of SOM230 and OCT binding affinity profiles for sst_5, it seems unlikely that OCT, binding 40-fold less to sst_5 compared with SOM230, would exert such a strong and long lasting insulin inhibition via sst_5 subtype. Therefore, these seemingly discrepant effects of OCT and SOM230 on insulin levels, clearly suggest a dominant role for sst_2 in regulating human insulin secretion. As glucagon release appeared far less sensitive for inhibition by SOM230 as compared with octreotide (44-fold) in several *in vivo* animal models, and because peripheral glucagon measurement does not represent pancreatic glucagon (gastro-intestinal glucagon is measured as well) concentrations, peripheral circulating glucagon levels were not analyzed in our study. Moreover, cumulating data suggest that post-absorptive plasma glucose concentrations are maintained within the physiological range by insulin alone, whereas glucagon only becomes relevant when glucose drifts below the physiological range. Therefore, we hypothesized whether SOM230 might alter glucose metabolism via extra-pancreatic mechanisms, whereby the expression of sst in target tissues of insulin action, i.e. liver, visceral fat and skeletal muscle seems a prerequisite for this theory. By quantitative PCR we indeed detected the expression of sst_1 and sst_2 in these tissues (**chapter III-3** and **chapter III-4**). It is well known that insulin increases glucose uptake in muscle and fat. Based on sst_1 and sst_2 binding affinity differences between SOM230 and OCT, i.e. a 30-fold higher and 2.5-fold lower affinity, respectively, and the differential effects of these SRIF-analogs on glucose homeostasis, we hypothesized that sst_1 alone or in combination with sst_2, may be involved in SOM230-mediated effects on insulin signaling by activation protein tyrosine phosphatases (PTP), which may catalyse the rapid dephosphorylation of the insulin receptor (IR) and the insulin receptor substrates (IRS). This would also explain why OCT 100µg did not induce a profound glucose release, because this OCT dosage is by far not sufficient to activate sst_1 because

of very low sst_1 binding affinity (EC_{50} >100 nM). SOM230, via sst_1 activation, might increase PTP activation, which subsequently could result in IR- or IRS-dephosphorylation. Via such a mechanism, e.g. attenuating the insulin-signaling cascade, SOM230 may even induce a relative state of insulin resistance. However, our experiments in Huh-7 hepatoma cells, which were shown to express only sst_1 and sst_2 at the mRNA level, do not demonstrate that sst_1 or sst_2 activation can attenuate tyrosine phosphorylation at the IR itself. Possibly, attenuation of insulin signaling by SOM230 could also take place at the level of IRS-1or IRS-2 proteins. Both IRS-1 and IRS-2 knockout mice exhibit insulin resistance while IRS-3 and IRS-4 knockout mice have a normal metabolism. Therefore, further studies are required to evaluate whether insulin-induced phosphorylation of IRS-1 and/or IRS-2 can be altered through sst_1 and/or sst_2 activation by SOM230.

In conclusion, our *in vitro* and *in vivo* data suggest that SOM230 has the potency to increase the number of acromegalic patients which can be biochemically controlled during long-term medical treatment. While the sst_2 seems to be the dominant receptor in controlling GH-hypersecretion in acromegaly, SOM230 can mediate its suppressive actions via sst_2 and, in contrast to OCT, seems also to mediate a GH-suppressive effect via the sst_5 receptor. It should be noted, however, that in one patient OCT was far more efficacious compared with both dosages SOM230 to lower GH levels. Until now, this feature remains to be clarified. Whether higher dosages of SOM230 in this particular case would indeed induce similar lowering actions on GH concentrations as seen by OCT, remains uncertain. Future studies will have to address the question whether SOM230 treatment is able to control pituitary adenoma size as well. Besides sst_2 and sst_5, also sst_1 and sst_3 seem to be involved in inhibition of cell proliferation and in the induction of apoptosis. This suggests that SOM230, with good affinity for both sst_1 and sst_3, might have potential anti-proliferative and tumor size reducing effects as well. Long-term treatment with SOM230 will also elucidate whether this drug can endure its advantages over OCT to control GH and IGF-I hypersecretion. Moreover, although SOM230 was well tolerated and caused no serious side effects, the alteration of glucose

homeostasis needs further evaluation. It seems of extreme importance for future clinical application of SOM230 to evaluate whether the elevated glucose levels will diminish during long-term treatment, and moreover, to elucidate the metabolic pathways behind this hyperglycaemic effect.

As already discussed, the inhibitory effects of sst_2 and sst_5 activation in the regulation of GH secretion *in vitro* and *in vivo* in patients with a GH-secreting pituitary adenoma, are supportive for a dominant role of sst_2 in the regulation of GH release. The data from the study described in **chapter IV-1**, provide a first quantitative evidence to support the concept that sst_2 is the functional dominant receptor subtype over sst_5. In these studies, a cAMP Response Element-Luciferase Reporter Gene Assay and [^{125}I-Tyr^{11}]-SRIF-14 radioligand binding studies were used in HEK 293 cells transiently co-transfected to express different ratios of sst_2/sst_5. The efficacy of OCT appeared not to be affected by the different sst_2/sst_5 expression ratios, whereas the sst_5-preferring SRIF-analog BIM-23206 was only maximally effective if the sst_5 expression level was superior to sst_2 expression. The multiligand SOM230 appeared far less sensitive for the ratio of sst_2/sst_5 expression due to its good binding affinity for sst_2. Apparently, in the transfected cell system a good correlation between membrane binding affinity and functionality for individual sst subtypes is found. However, when two sst subtypes are co-expressed, a functional interplay, *i.e.* dominance by sst_2 over sst_5, is observed. In addition, the presence of the sst_2 antagonist BIM-23454 the effect of OCT drifts towards its low sst_5 binding affinity in sst_2+sst_5 expressing cells, whereas a subnanomolar binding affinity is suggested to be physiologically active. These data confirm that OCT is not effective when sst_2 is expressed at a low level, i.e. cannot lower GH in the subgroup of acromegalic patients that are (partially) non-responders for OCT. Our experiments with the sst_2 antagonist BIM-23454, moreover, demonstrate that SOM230 can still be effective when sst_2 availability is low (as in those acromegalic patients in which OCT is ineffective), because it can now function via sst_5 receptors for which it endorses a superior sub-nanomolar binding affinity. A critical reflection regarding the studies in HEK 293 cells expressing differ-

ent ratios of sst_2/sst_5, reveals that up till now no intracellular regulatory mechanism is known that can account for the functional dominance of sst_2. Studies which describe GPCR signal transduction, especially with respect to individual sst subtype binding to G-proteins, seem crucial to understand the physiological rationale behind the hypothesis of sst_2 dominance. The availability of G-proteins, however, does not seem to be a limiting factor in the experimental design we have used. This is supported by the observation that the efficacy of the G-protein coupled Dopamine D2 receptor (D2R) analog Cabergoline to inhibit adenylyl cyclase activity is not affected when D2R are being expressed either alone or co-expressed with sst_2 and sst_5 receptor subtypes in HEK 293 cells. Still, further understanding of the stoichiometry of ligand, receptor and G-protein, by GTPγS binding-, co-immunoprecipitation- and/or fluorescence tagged receptor- studies can be of experimental help in this matter. In recent years, the use of RNA interference has been a new approach to manipulate gene expression in mammalian systems. This technique could also be potentially very suitable to ultimately investigate further the functional role of the endogenously expressed sst_2 and sst_5 in the regulation of GH release from primary cultures of GH-secreting pituitary adenoma cells. In **chapter IV-2**, we demonstrated that a synthesized siRNA constructs for the sst_2 and sst_5 gene have merit to become such novel tools. At present, the transfectability of cells is the limiting step in siRNA-mediated gene silencing, especially in primary cells which are notorious for having low transfection efficiencies when plasmid DNA is used. Furthermore, several aspects of RNAi, i.e. non-specific silencing effects and activation of the interferon response may occur when endogenous (sst-) expressing mammalian cell systems are used. Chemical features of dsRNAs, as well as their expression levels and delivery routes, may determine whether they become visible to the interferon response machinery.

So far, we have confirmed that the sst_2-preferring SRIF-analog OCT is effective in approximately 65% of acromegalic patients to lower circulating GH concentrations, but it seems largely dependent upon a certain expression level of sst_2 on the pituitary adenoma cells. The multiligand SOM230,

with good and superior binding affinity for sst_2 and sst_5, respectively, seems less susceptible to the sst expression pattern on the tumor cells. Which characteristics should be incorporated when SRIF-analogs are designed for the treatment of acromegaly? An optimal SRIF-analog should have a very high affinity for both sst_2 and sst_5 receptor subtypes (in particular, the analog should have an affinity close or better than that for sst_2). If not, for example when an analog with a high affinity for sst_5 and low affinity for sst_2, this analog will "suffer" from the presence of the dominant sst_2 receptor for which it has low affinity. Future studies should also be performed to clarify how the functional dominance of sst_2 is actually being executed at the cellular level. One explanation might be that sst subtypes display differential binding affinities for the available G-proteins within the cytoplasm, and that the regulation of the expression and activity of one type of G-protein coupled receptor in a given cell type would inevitably affect the ligand-sensitivities of other co-existing G-protein coupled receptors.

Somatostatin receptor subtypes in Cushing's disease

The endogenous Cushing syndrome is predominantly caused due to excess ACTH release from a ACTH-secreting pituitary adenoma, also known as Cushing's disease (CD). Since significant mortality and morbidity accompany this condition, proper medical intervention is necessary. Transsphenoidal surgery is the treatment of choice for pituitary-dependent CD. Although transsphenoidal surgery allows cure of CD, the reported success rates vary between 50 to 90%. If surgery fails radiotherapy, either alone or in combination with adrenolytic agents, may be used. Unfortunately, none of the current treatment modalities ensure a full and permanent cure, as the rate of recurrence of the disease, depending on the criteria of initial cure, varies between 5 to 24% in the literature. Therefore, physicians have explored new medical strategies, preferably based on fundamental and (patho-) physiological pathways, with the hope to increase the chances of curation in this group of patients. Neuromodulatory agents, such as dopamine and SRIF, have been proposed to be of therapeutic interest in the medical treatment of CD. The conclusion of various case reports,

however, is that the sst_2-preferring analog OCT is ineffective in treating CD. **Chapter V** is devoted to the functional role of sst in the patho-physiological regulation of ACTH release in CD. In **chapter V-2** glucocorticoid treatment induced remarkable differences with respect to the role of sst_2 and sst_5 in regulating ACTH release in mouse corticotroph AtT-20 cells, predominantly expressing sst_2 and sst_5. In the absence of dexamethasone (DEX), OCT and SOM230 potently inhibited CRH-induced ACTH release, while the sst_5-specific analog BIM-23268 appeared the to be the least potent. In the presence of DEX, a physiological nanomolar concentration of SOM230 and BIM-23268 still inhibited CRH-induced ACTH release whereas the suppressive effects of OCT were almost completely blocked, and in addition, the IC_{50} values for OCT and BIM-23268 during DEX treatment shift toward their sst_5 binding affinity. The high potency for SOM230 was not affected by DEX. These data suggest down-regulation of sst_2 by DEX, while sst_5 receptors seem more resistant to DEX. This was indeed confirmed by the mRNA as well as by the sst membrane binding studies. These data suggest that in untreated patients with CD the expression level of sst_2 is too low for OCT to lower ACTH and cortisol level. Indeed, as shown in **chapter V-1**, relatively low levels of sst_2 mRNA in a series of primary human corticotroph adenomas of patients with CD were found. On the other hand, we found a predominant expression of sst_5 mRNA in corticotroph adenomas. This sst mRNA profile in human corticotroph adenomas, i.e. a low sst_2 and a significant sst_5 mRNA expression, supports the concept that glucocorticoids down-regulate sst_2 expression in human corticotroph adenomas while sst_5 receptors are more resistant to glucocorticoid pressure. These observations also support the lack of efficacy of OCT in lowering circulating ACTH and cortisol levels in these patients, because sst_2 expression is too low and sst_5-membrane binding affinity by OCT is not high enough to make OCT therapeutically active in patients with CD. Support is formed by SOM230 induced inhibition of basal and CRH-stimulated ACTH release by the corticotroph adenomas, which appeared comparable with the observations in AtT20-cells (**chapter V-2**), *i.e.* significant inhibition by SOM230 compared with OCT, even in the presence of DEX. SOM230 did not inhibit AtT20 cell-

proliferation or POMC synthesis during a 72 hr incubation *in vitro*. Therefore, increased ACTH breakdown may form an additional explanation for the inhibitory effect of SOM230 (and SRIF) on basal ACTH secretion.

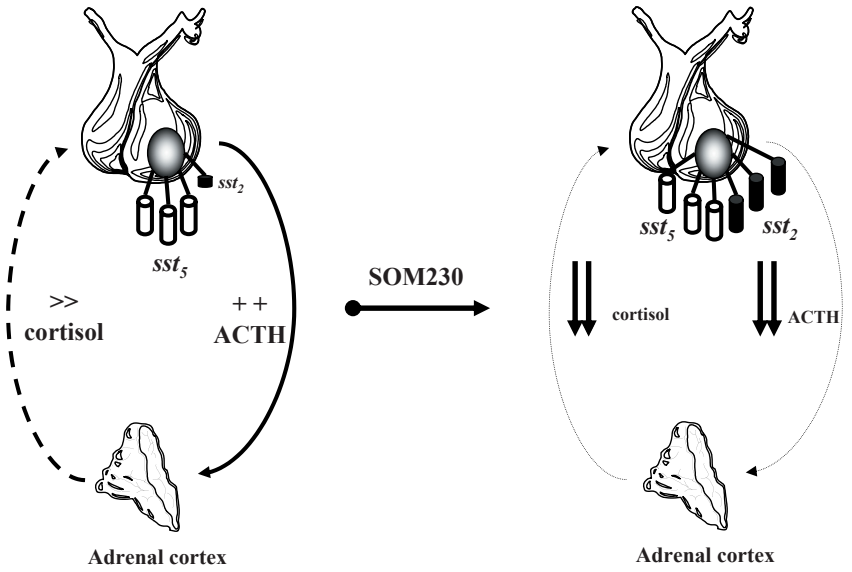

Figure 1. SOM230 in untreated pituitary-dependent Cushing's disease. A de novo human corticotroph adenoma has predominant expression of sst_5 and low expression of sst_2. The elevated ACTH and cortisol levels down-regulate sst_2 expression on the tumor, making it almost impossible for the current clinically available sst_2-preferring SRIF-analogs to inhibit ACTH and cortisol secretion (left panel). SOM230, however, through activation of sst_5, should be able to lower ACTH and cortisol levels. In this relative hypocortisolemic state, the down-regulation of sst_2 by cortisol might be abrogated. Subsequently, because SOM230 also bind with good affinity to sst_2, enhanced suppression of ACTH and cortisol via restored sst_2 expression becomes suggestive (right panel).

In conclusion, we propose that sst_2 + sst_5 preferring SRIF-analogs, such as SOM230, might become of therapeutic interest in CD. The suppression of ACTH levels by activation of sst_5 in patients with CD might lower cortisol levels. Since cortisol lowers sst_2 expression, these suppressive effects might subsequently be (partially) abrogated and enhanced ACTH inhibition via restored sst_2 expression becomes suggestive. Therefore, prolonged treatment with SOM230 may be able to lower ACTH levels in CD even more, because it could now function via both sst_5 and sst_2 receptor subtypes (Fig. 1). An open label phase II trial in nine patients with untreated or recurrent CD has demonstrated promising results with SOM230. After two

weeks of SOM230 treatment, 600µg s.c. twice daily, free urinary cortisol levels normalized in three patients, while in the remaining six patients urinary cortisol levels were suppressed by 17-60%. Prolonged treatment shall reveal whether this multiligand can indeed be of clinical potential for this serious neuro-endocrine condition. On the other hand, glucose intolerance in all patients studied was documented during this short treatment period, and one patient even withdrew from the trial due to overt diabetes mellitus. Even though patients with CD are already known to suffer from glucose intolerance, it seems crucial for the future clinical development of SOM230 to retrieve answers with respect to this serious side effect.

Tachyphylaxis and chimeric compounds in neuro-endocrine diseases

Chapter VI-1 is a review of the clinical potentials and possible pitfalls of novel sst subtype specific analogs, as well as universal binding SRIF-peptides in sst-positive neuro-endocrine tumors. Although the current clinically available sst_2-preferring analogs OCT and Lanreotide effectively control hormonal hypersecretion by GH-secreting pituitary adenomas, islet cell tumors, and carcinoids, significant differences are observed among patients with respect to the efficacy of treatment. In striking contrast to the absence of the occurrence of adaptation or tachyphylaxis of hormone secretion by continuous exposure to OCT or Lanreotide in patients with GH-secreting pituitary adenomas, are the observations in patients with islet cell tumors or carcinoids. In the majority of patients with metastatic carcinoids, VIPomas, gastrinomas, insulinomas and glucagonomas, treatment with OCT induces a rapid improvement of clinical symptomatology. On the other hand, the majority of the patients show tachyphylaxis of the inhibition of the secretion of tumor related hormones by OCT within weeks to months. This effect may be initially reversed by increasing the dosage of OCT, but eventually the drug becomes ineffective in all patients. The potential mechanisms responsible for this tachyphylaxis are not known at present. Multiple causes may underly the development of tachyphylaxis to octapeptide SRIF analogs treatment in patients neuro-

endocrine gastro-enteropancreatic tumors. These include: 1. Differential expression of sst subtypes among tumors. While a preferential expression of sst_2 and sst_5 is found in GH-secreting pituitary adenomas, expression of sst_1 and sst_3 in combination with the presence of sst_2 in other neuro-endocrine tumors is observed. 2. Sst receptor induced down- and/or up-regulation; 3. Outgrowth of clones of tumor cells that lack sst receptors and 4. Mutations in sst genes leading to absence of functional receptor proteins. Since one of the potential mechanisms of OCT-resistance includes desensitization and/or down-regulation of sst, we have investigated this issue in sst_2 and sst_5 over-expressing HEK 293 cells. Much of the current (pre-) clinical knowledge about tachyphylaxis is built upon sst_2-mediated mechanisms and sst_2-preferring analogs. Therefore, it seems of interest to compare OCT with novel SRIF-analogs, having a different sst binding profile compared with OCT, to evaluate whether they may be of potential interest for the medical treatment of (OCT-resistant-) neuro-endocrine tumors. In **chapter VI-2,** we evaluated tachyphylaxis of sst_2 and sst_5 subtypes expressed alone or together in HEK 293 cells to various SRIF-analogs. Prolonged (24h) pre-treatment with OCT resulted in a nearly total loss of responsiveness of sst_2 receptor expressing cells in terms of inhibition of adenylyl cyclase activity. This observation may provide one of the explanations for the *in vivo* data in patients with neuro-endocrine tumors which are prone for developing unresponsiveness for OCT after prolonged treatment. What seems interesting is that SOM230, binding with a 2.5 lower binding affinity to sst_2 compared with OCT, induces a loss of responsiveness of sst_2 that can be classified as partial tachyphylaxis of SRIF-analog induced inhibition of adenylyl cyclase activity. In addition, the sst_5 also seems less susceptible to tachyphylaxis compared with the sst_2 subtype. SOM230 pre-treatment, which has a superior binding affinity for sst_5 resulted in a significant lower induction of tachyphylaxis in sst_5-expressing cells compared with the (almost) complete tachyphylaxis induced by OCT in sst_2-expressing cells. Moreover, no loss of sensitivity of sst_5 was found if cells were pretreated with OCT. These data merely indicate that the sst_5 subtype seems less susceptible to tachyphylaxis after prolonged treatment with a sst_5-preferring analog, and suggest that

the sst_5 receptor might form a potential new target for the treatment of OCT-resistant neuro-endocrine tumors. Also, prolonged treatment with SOM230 might result in a longer duration of action, because SOM230 induces only partial tachyphylaxis of both the sst_2 and sst_5. However, as already discussed, it should be taken into account that the expression levels of sst_5 in carcinoid tumors is not as high as in GH-secreting pituitary adenomas and that other sst subtypes, for example sst_1, are profoundly expressed in carcinoid tumors as well. Furthermore, the mechanism behind distinct agonist-induced variability of receptor tachyphylaxis remains to be elucidated. Although SRIF-analog induced down-regulation of sst_2 and sst_5 mRNA expression levels was observed in our experiments, which appeared not to be statistically different between various SRIF analogs, we believe that other processes like G-protein uncoupling and/or endocytosis might play a role in tachyphylaxis to SRIF-analogs. Additional studies, therefore, are inevitably required to determine which cellular mechanisms are actually triggered after the distinct agonist-induced receptor tachyphylaxis. Finally, the potencies and efficacies of SOM230 and OCT after pre-treatment with either OCT or SOM230 in the sst_2 + sst_5 co-transfected cells to inhibit adenylyl cyclase activity fully reflected the resultant of the SRIF-analog induced tachyphylaxis in the mono-transfected state. Extrapolating these latter observations to the clinical setting of a patient diagnosed with a carcinoid tumor, expressing both sst_2 and sst_5, prolonged OCT treatment may result in complete tachyphylaxis of the sst_2 subtype, but the sst_5 will remain functionally active. Therefore, it won't be of any use for subsequent treatment with a sst_2-selective or preferring SRIF-analog, but adjuvant treatment with SRIF-analogs that can bind with sub-nanomolar affinity to sst_5 might be of interest to elicit once again a therapeutic response in these OCT-resistant patient. On the other hand, prolonged treatment with SOM230 might result in a longer duration of action irrespective of the sst_2 and sst_5 expression levels, because SOM230 induces only partial tachyphylaxis of both receptor subtypes.

As already discussed extensively, acromegalic patients that are classified as only partially responsive to long-term therapy with OCT and

Lanreotide represent approximately 60% of the population. Dopamine agonists, however, were the first drugs used in the medical treatment of acromegaly. The efficacy of dopaminergic treatment increased to reach 35% of patients with the advent of the high affinity Dopamine D2 receptor (D2R) analog, cabergoline (CAB). In selected acromegalic patients, the combination of SRIF- and D2R-analog treatment has been shown to be more effective than treatment with the individual analogs. Moreover, under experimental conditions sst_5 and D2R have been demonstrated to heterodimerize in the presence of appropriate ligands, which provided a rationale for the development of novel molecules which might enhance biochemical control during long-term medical treatment of acromegalic patients. Indeed, the hybrid 'dopastatin' molecule, BIM-23A387 which has high binding affinity for both sst_2 and D2R, has an enhanced inhibitory effect on *in vitro* PRL and GH release from human pituitary adenoma. This chimeric molecule appeared more potent than either sst_2 or D2 selective analogs alone and interestingly, no additivity was even found when the sst_2 and D2 selective analogs were added simultaneously to primary cultures of GH-secreting pituitary adenoma cells. This enhanced potency of BIM-23A387 (EC_{50} was 50 times lower than that of the individual sst_2 and D2 agonists as well), however, could not be explained on the basis of the binding affinity of the compounds for sst_2 and D2 receptors. It has been suggested that GH suppression by BIM-23A387 is not mediated via either individual sst_2 or D2 receptors, but requires a functional interaction between the two receptors in which the D2 receptor might have a dominant role. More recent studies with novel chimeric compounds, with differing enhanced activity at sst_2, sst_5 and D2R, demonstrated that these molecules consistently produced significantly greater suppression of GH and PRL than either OCT or single-receptor-interacting ligands in tumors from patients classified as only partial responsive to OCT therapy. Although the higher efficacy of the chimeric compounds targeting sst_2, sst_5 and D2R seems to be at least partially linked to their higher efficacy for sst_2, other mechanisms by which such molecules produce enhanced inhibition of GH secretion are lacking. Another explanation for the greater potency of the multi-receptor-interacting chimeric molecules may simply

be the fact that they can bind and activate multiple receptors. This ability increases the chance that once the ligand is released form one receptor it will rapidly occupy another receptor. To gain additional insights with respect to the functional explanation for the potency of these novel chimeric compounds, BIM-23A760, targeting sst_2, sst_5 and D2R, was evaluated in **chapter VI-3** for its efficacy to inhibit forskolin-induced adenylyl cyclase activity in sst_2+sst_5+D2R transfected mammalian cells. No differences in efficacy between BIM-23A760 and the combination of the sst_2-preferring analog BIM-23023 and the D2R-analog BIM-53097 or CAB, in terms of inhibition of adenylyl cyclase was found when all three receptor subtypes where co-transfected in HEK 293 cells. Moreover, although BIM-23A760, compared with BIM-23023, has a 10-fold higher binding affinity for sst_2 and a 10-fold lower binding affinity for sst_5, no differences were observed in functional activity. In addition, although BIM-23A760 has a similar binding affinity for the D2R, compared with the internal control BIM-53097, the tri-chimeric molecule was 20-fold more potent to inhibit adenylyl cyclase activity in cells, expressing only the D2R. While it has been hypothesized that multiple G-protein coupled receptor ligands can induce receptor homo- and heterodimerization, which can result in a distinct novel G-protein coupled receptor with enhanced functionality compared with the individual receptor subtypes, the data with BIM-23A760 are not in favour of such an oligo-heterodimerization between sst_2, sst_5 and D2R. We propose, in addition to the current knowledge about GPCR function, that dopastatin chimeras can alter ligand-mono-receptor complexes differently, allowing prolonged stabilization of its active conformation or alteration of the rate of internalization. These molecules should have superior binding-affinity towards the sst_2, sst_5 and D2R, thus initiating a super-agonist ligand-receptor conformation for each individual receptor. Eventually, such a hybrid compound may form an ideal pharmacological tool to treat GH hypersecretion, independent of the varying sst and D2R expression levels on the pituitary adenomas. Additional experiments, with the use of sst- and D2 receptor analogs and antagonists, have to be carried out in order to understand how these chimeric molecules actually work at the cellular level. Resonance Energy transfer (FRET/BRET)

studies, confocal microscopy and RNAi techniques, seem crucial to understand how individual, and possibly combined, sst and D2R undergo ligand-induced G-protein coupled receptor trafficking in mammalian cells.

In summary, the novel multiligand SRIF-analog SOM230 has been shown to have potential as a novel medical treatment option for acromegalic patients. Whereas OCT mediates its GH-suppressive actions predominantly via sst_2, SOM230 can lower hormonal hypersecretion from GH-secreting pituitary adenomas via both sst_2 and sst_5 and, therefore, it has an advantage over OCT which seems beneficial in the medical treatment of about two thirds of acromegalic patients. With respect to the interplay between the two main key players in the regulation of GH-secretion from GH-secreting pituitary adenomas, the sst_2 subtype seems to function in a dominant way over sst_5 to inhibit adenylyl cyclase activity. In mouse ACTH-secreting pituitary adenoma cells the sst_5 subtype, as compared with sst_2, has been demonstrated to be glucocorticoid resistant, which is supported by the high sst_5 mRNA and low sst_2 mRNA expression in primary cultures of human ACTH-secreting pituitary adenomas. In addition, SRIF-analogs targeting sst_5 and/or sst_2+sst_5 can lower ACTH secretion from mouse and human ACTH-secreting pituitary adenomas more effectively compared with OCT. Since these superior inhibitory effects of sst_5-targeting analogs on ACTH release remain unaffected in the presence of glucocorticoids, sst_5-preferring SRIF analogs may have merit for the medical treatment of Cushing's disease. Evaluating sst_2 and sst_5 subtypes, expressed alone or together in HEK 293 cells, for tachyphylaxis to various SRIF-analogs demonstrated that prolonged pre-treatment with OCT results in a nearly total loss of responsiveness of the sst_2 receptor to inhibit adenylyl cyclase activity What seems interesting is that SOM230 induces a loss of responsiveness of sst_2 that can be classified as partial tachyphylaxis of SRIF-analog induced inhibition of adenylyl cyclase activity. In addition, the sst_5 also seems less susceptible to tachyphylaxis compared with the sst_2 subtype, indicating that the sst_5 subtype seems less susceptible to tachyphylaxis after prolonged treatment with a sst_5-preferring analog, suggesting

that the sst_5 receptor might form a potential target for the treatment of OCT-resistant neuro-endocrine tumors. Finally, one of the latest pharmaceutical novelties for the medical treatment of sst-expressing neuro-endocrine tumors, the novel chimeric compound BIM-23A760, targeting $sst_{2,}$ sst_5 and D2R, was not shown to be more effective to inhibit adenylyl cyclase activity as compared with the individual receptor-ligands. It is suggested, therefore, that dopastatin chimeras can alter ligand-mono-receptor complexes differently, allowing prolonged stabilization of its active conformation or alteration of the rate of internalization.

Chapter VIII

SUMMARY-SAMENVATTING

The main subject of the current thesis is the role of somatostatin (SRIF) receptors subtype 2 and 5 in the patho-physiological regulation of GH and ACTH release from human pituitary adenomas. The sst expression pattern in both types of pituitary adenomas is evaluated with respect to the inhibitory effects by SRIF-analogs on hormone secretion from the primary cultured tumor cells. The novel multiligand SOM230, compared with the current clinically available SRIF-analog OCT, is evaluated both for its clinical potential in the medical treatment of acromegaly, as well for *in vitro* for its potential in the medical treatment in patients with Cushing's disease. SOM230, together with sst-selective analogs and chimeric molecules, targeting the sst_2, sst_5 and Dopamine D2 Receptor, are used as tools to retrieve insights with respect to the functional interplay between sst_2, sst_5 and D2R in the regulation of adenylyl cyclase activity as well the susceptibility of sst receptors to undergo ligand-induced adaptation, *i.e.* tachyphylaxis, of inhibition of adenylyl cyclase activity by SRIF-analogs.

In **chapters III-1** and **III-2**, the role of SRIF receptor subtypes in the regulation of *in vitro* and *in vivo* GH release by acromegalic patients was evaluated. The inhibitory effect of the novel multiligand SOM230, which binds with high affinity to four of the five human sst, on hormone release by GH-secreting pituitary adenomas are compared with the clinically used sst_2-preferring analog Octreotide (OCT). All GH-secreting pituitary adenomas that were studied showed mRNA expression for sst_2 and sst_5. Whereas the relative sst_5 mRNA expression level in the GH-secreting pituitary adenomas was higher compared with sst_2 mRNA expression, the sst_2 subtype seems to be a dominant receptor regulating *in vivo* and *in vitro* GH secretion in acromegaly. This conclusion is based on the observation that the inhibitory effects of OCT and SOM230 on GH release from the pituitary adenomas was positively correlated with the levels of sst_2 mRNA expression, but not with sst_5 mRNA expression, of the tumors. Moreover, during the first proof-of-concept study with SOM230, a concentration-dependent inhibitory effect of SOM230 on circulating GH concentrations from patients with acromegaly supports a dominant role of sst_2 in the regulation of GH release, since the 100μg concentration of SOM230 was

not as effective as 100µg OCT, to suppress GH release *in vivo*. Regarding the superior binding affinity of SOM230 for the sst_5 subtype and the comparable superior binding affinity of OCT for sst_2 receptors, it could have been expected that 100µg SOM230 acting via sst_5, would be as potent as 100µg OCT, exerting its action via sst_2, to suppress GH release. The results demonstrated that only 250µg SOM230, compensating for the 2.5 lower binding affinity for sst_2 compared with OCT, was as effective as 100µg OCT to suppress GH secretion in acromegalic patients. On the other hand. however, only SOM230, in striking contrast to OCT, can lower GH concentrations when sst_2 expression was low and sst_5 expression was high on the adenoma cells. This is likely caused by the sst_5 binding affinity of SOM230, which is 40-fold higher as compared with OCT. The multiligand SOM230, therefore, has a clear advantage over OCT with the potential to increase the number of acromegalic patients that can be biochemically controlled during long-term medical treatment. SOM230, in contrast to OCT, appeared to lower PRL secretion in prolactinomas as well, which was clearly related to the level of sst_5 expression on the adenoma cells. Additional analysis regarding the effects of SOM230 on several metabolic parameters in acromegalic patients *in vivo* are described in **chapter III-3**. Both dosages SOM230 inhibited free IGF-I more sustained as compared with OCT. Circulating IGFBP-1 is capable of binding free IGF-I, thereby lowering the metabolic actions of free IGF-I. The regulation of IGFBP-1 is suggested to be regulated directly via SRIF-analogs. The superior action of OCT compared with a lower effectiveness of SOM230, which has a 2.5 lower affinity for sst_2, in stimulating IGFBP-1 levels, supported the concept of direct regulation of IGFBP-1 by SRIF-analogs via sst_2. Comparable elevations of glucose concentrations during lunch were observed after SOM230 and OCT administration. However, 250µg SOM230 elicited an acute increase in glucose levels 1 h post-injection. This effect could not be explained by its concomitant slight inhibitory effect on insulin secretion. Since absolute insulin concentrations were not dramatically affected by SOM230, it was hypothesized that this multiligand negatively influences insulin action, *i.e.* induces a state of insulin resistance, in peripheral target tissues such as liver,

visceral adipose tissue and skeletal muscle. Subsequently, as shown in **chapter III-3** and **III-4**, the observed sst_1 and sst_2 mRNA expression pattern in peripheral target tissues of insulin action, forms a prerequisite for the involvement of sst subtypes in the regulation of insulin action. The evaluation of insulin receptor (IR) auto-phosphorylation in the human hepatoma cell line HuH-7, endogenously expressing sst_1 and sst_2, did not demonstrate that SOM230 can attenuate IR autophosphorylation by possible activation of protein tyrosine phosphatases. Other causes, for instance dephosphorylation of the IRS proteins, which are more downstream of the IR, by SOM230 via sst_1 and/or sst_2 remains to be clarified

Using [^{125}I-Tyr^{11}]SRIF-14 radioligand binding and functional cAMP response studies in transiently transfected HEK 293 cells expressing different sst_2/sst_5 ratios, **chapter IV-1** provides support for a dominant role of sst_2 over sst_5 in the inhibition of adenylyl cyclase activity. The lowering of intracellular cAMP concentrations via inhibition of adenylyl cyclase activity is one of the main second messenger systems involved in inhibition of GH-secretion. This fundamental approach to evaluate whether a functional interplay between sst_2 and sst_5 occurs, is fully in accordance with the *in vitro* and *in vivo* correlation of sst_2 mRNA expression levels, but not sst_5 mRNA expression, with the inhibitory effects of OCT and SOM230 on GH release from GH-secreting pituitary adenomas. Experiments with a sst_2-antagonist, are supporting the clinically observed advantage of SOM230 compared with OCT in those acromegalic patients classified as (partially) OCT-resistant. In the presence of the sst_2 antagonist, only SOM230 was still effective to suppress adenylyl cyclase activity in sst_2 + sst_5 expressing HEK 293 cells. Apparently, when sst_2 expression is low, OCT cannot be sufficiently effective via sst_5, because it's binding affinity for the sst_5 subtype is 40-fold lower compared with SOM230. In agreement with our hypothesis based on clinical observations, these fundamental studies support the concept that SOM230 can be beneficial in the medical treatment of acromegaly, because the multiligand can effectively lower GH release, as well as adenylyl cyclase activity, via both sst_2 and sst_5. RNA interference seems an interesting methodological novel tool al-

lowing silencing a specific gene of interest. **Chapter IV-2** shows that the silencing RNA constructs for the sst_2 and sst_5 gene, evaluated in several mammalian cell lines expressing either sst_2 or sst_5, have merit to become novel tools to eventually evaluate further the individual role of sst subtypes in the regulation of hormone (hyper-) secretion from endogenously sst-expressing primary pituitary adenoma cells.

Chapter V describes the sst expression pattern in pituitary adenomas from patients with Cushing's disease (CD), as well as the functional characterisation of sst_2 and sst_5 in regulating ACTH release. In patients with untreated CD, OCT has not been effective to lower circulating ACTH and cortisol levels, which has been suggested to be caused by down-regulation of sst_2 receptors by glucocorticoids. In mouse corticotroph AtT-20 cells, a down regulation of sst_2 mRNA and protein levels by glucocorticoids was indeed observed, which supports the clinical observation that OCT is ineffective in lowering ACTH and cortisol levels in patients with untreated CD. The suppressive effects on ACTH release in AtT-20 cells and primary cultures of human corticotroph adenomas and the observation that sst_5 appeared to be relatively resistant to down-regulation by glucocorticoids, suggests that this sst subtype plays an important role in the regulation of (tumoral) ACTH release. As the sst_5 seemed the predominantly sst expressed in human corticotroph adenomas, whereas low sst_2 mRNA expression levels support once more the lack of efficacy of OCT in these tumors, it is hypothesized that SRIF-analogs targeting sst_5 and sst_2, or even universal SRIF-analogs, may become a new medical treatment option to the control of ACTH and cortisol hypersecretion in untreated patients with pituitary dependent CD.

In **chapter VI-1** an overview is given with respect to the role of novel SRIF-analogs in the treatment of neuroendocrine tumors. A potential medical problem during prolonged treatment with the sst_2-preferring SRIF-analogs OCT and Lanreotide is tachyphylaxis to the initial inhibitory effects of different SRIF-analogs. Therefore, this tachyphylaxis of the inhibitory effects of SRIF-analogs on adenylyl cyclase activity in a mammalian cell

line expressing sst_2, sst_5 or co-expressing sst_2 and sst_5, is investigated in **chapter VI-2**. HEK 293 cells have been transiently transfected with sst_2 and/or sst_5 subtypes and treated for a prolonged period of time with several SRIF-analogs. Prolonged OCT-treatment, in accordance with the clinical experience in carcinoids tumors, results in a complete tachyphylaxis of cAMP formation via the sst_2 subtype. In contrast to OCT, SOM230 and BIM-23023, the latter being a sst_2-specfic agonist, induced a loss of responsiveness of sst_2 that can be classified as partial tachyphylaxis of inhibition of adenylyl cyclase activity. On the other hand, the sst_5 was demonstrated to be less susceptible to tachyphylaxis compared with the sst_2 subtype. This might indicate that targeting the sst_5 could form a potential treatment strategy in patients with a neuro-endocrine tumor which has become unresponsive after prolonged treatment with current sst_2-preferring SRIF-analogs. In addition, on the basis of this agonist-selective induction of tachyphylaxis of a single sst subtype, it is suggested that the prolonged activity of agonists with very good binding affinity for sst_2 and/or sst_5 differentially elicit tachyphylaxis of both the sst_2 and the sst_5 receptor, presumably due to distinct agonist-induced receptor conformations. Representing one of the potential novel medical tools for the treatment of neuroendocrine tumors including pituitary adenomas, BIM23A760, targeting both sst_{2+5} and dopamine D2 (D2R) receptors has been critically evaluated in a series of sst_2+sst_5+D2R transfected mammalian cells. This is described in **chapter VI-3**. The functional efficacy of BIM-23A760 in the experimental model seems not in favour of oligo-heterodimerization between sst_2, sst_5 and D2R. The efficacy of BIM-23A760, compared with the mono-receptor sst ligands and the D2R agonist Cabergoline, in mono-transfected cells with one of the three target receptor subtypes to inhibit adenylyl cyclase activity is not different as compared with their efficacy in HEK 293 cells transfected with all thee receptor simultaneously. Also, even though BIM-23A760 and its D2R-targeted internal control BIM-53097 share comparable binding affinities for D2R, a more potent inhibition of adenylyl cyclase activity by BIM-23A760 was observed. Therefore, it is suggested that the dopastatin chimera BIM-23A760 can alter ligand-mono-receptor complexes differ-

ently compared with the individual mono-receptor ligand, that already can result in enhanced potency of this novel pharmacological agent.

In **chapter VII** the results of the studies reported in this thesis are discussed, as well as the possible clinical significance and potential future developments of using novel sst subtype selective or universal SRIF-analogs.

Het hoofddoel van dit proefschrift is geweest om meer inzicht te krijgen over de rol van somatostatine (SRIF) receptor subtype (sst) 2 and 5 in patho-physiologische regulatie van GH en ACTH secretie door humane hypofyse adenomen. Het sst expressie patroon in beide type hypofyse tumoren is onderzocht met betrekking tot de remmende werking van SRIF-analogen op de hormoon productie door hypofyse adenoom cellen. Het nieuwe multiligand SOM230, afgezet tegen het klinisch beschikbare SRIF-analoog Octreotide (OCT), is onderzocht voor zowel zijn klinische potentie voor de medische behandeling van acromegalie maar tevens ook voor zijn *in vitro* potentie voor de medicamenteuze behandeling van patiënten met de ziekte van Cushing. SOM230, tezamen met sst-selectieve analogen en chimerische moleculen, welke bindingsaffiniteit hebben voor sst_2, sst_5 en de dopamine D2 receptor (D2R), zijn verder gebruikt als hulpmiddelen om nieuwe inzichten te verkrijgen omtrent de functionele interactie tussen deze drie receptoren aangaande adenylyl cyclase activiteit alsmede voor het ondergaan van ligand-geïnduceerde adaptatie van sst, i.e. tachyphylaxie, voor remming van adenylyl cyclase activiteit door SRIF-analogen.

In de **hoofdstukken III-1 en III-2** is de rol van SRIF receptor subtypen in de regulatie van *in vitro* en *in vivo* GH secretie bij acromegalie patiënten onderzocht. De remmende werking van het nieuwe multiligand SOM230, wat met hoge affiniteit bindt aan vier van de vijf humane sst, op GH productie door GH-producerende hypofyse adenomen is vergeleken met het klinisch beschikbare sst_2-prefererende analoog OCT. In alle GH-producerende hypofyse adenomen is mRNA expressie van sst_2 en sst_5 aangetoond. De relatieve expressie van sst_5 mRNA in deze serie hypofyse adenomen bleek hoger te zijn dan de expressie van sst_2 mRNA, echter de sst_2 lijkt dominant over sst_5 voor wat betreft de *in vitro* en *in vivo* regulatie van GH secretie in acromegalie. Deze conclusie is gebaseerd op het feit dat de remmende werking van OCT en SOM230 op GH secretie door hypofyse adenomen positief gecorreleerd is met de mate van sst_2 mRNA expressie, maar niet met de mate van sst_5 mRNA expressie, van de tumoren. De observatie, tijdens de eerste klinische studie met SOM230 in acromegalie, dat SOM230 een concentratie-afhankelijk remmend effect

had op circulerende GH concentraties, ondersteunt de hypothese dat de sst_2 een dominante rol heeft in de regulatie van GH secretie, omdat 100µg SOM230 minder effectief bleek te zijn dan 100µg OCT. Omdat SOM230 en OCT een superieure bindingsaffiniteit voor respectievelijk sst_5 en sst_2 hebben, kon verwacht worden dat 100µg van iedere compound, via sst_5 of sst_2, even effectief zou zijn om GH secretie te remmen. Onze resultaten lieten zien dat alleen 250µg SOM230, waarmee gecompenseerd wordt voor de 2.5 lagere bindingsaffiniteit van SOM230 voor sst_2 vergeleken met OCT, even effectief was als 100µg OCT om GH secretie in acromegalie patiënten te remmen. Aan de andere kant, echter, bleek alleen SOM230 in staat te zijn om GH concentraties te verlagen als op de adenoom cellen de sst_2 expressie laag en sst_5 expressie hoog is. Dit wordt waarschijnlijk verklaard door de 40-voudige hogere bindingsaffiniteit, vergeleken met OCT, van SOM230 voor sst_5. De multiligand SOM230 heeft derhalve een duidelijk voordeel over OCT, met de potentie om het aantal patiënten dat biochemisch gecontroleerd kan worden tijdens langdurige medicamenteuze behandeling, te doen vermeerderen. Tevens bleek alleen SOM230, en niet OCT, in staat om PRL productie door prolactinoma cellen te remmen, wat duidelijk geassocieerd is met sst_5 mRNA expressie van de tumoren. Aanvullende analyse aangaande de effecten van SOM230 op verscheidende metabole parameters in acromegalie patiënten, zijn beschreven in **hoofdstuk III-3**. Beide doseringen SOM230 bleken vrije IGF-I spiegels meer te remmen dan OCT. Circulerend IGFBP-1 is in staat om vrij IGF-I te binden, waardoor de metabole werking van IGF-I wordt gereduceerd. De regulatie van IGFBP-1 is gesuggereerd direct gereguleerd te zijn door SRIF-analogen. De superieure stimulerende werking van OCT op IGFBP-1 concentraties, vergeleken met SOM230, ondersteunt het concept dat de directe regulatie van IGFBP-1 spiegels door SRIF-analogen is gemedieerd via sst_2. Vergelijkbare stijgingen in bloedsuikerspiegels tijdens en na de lunch zijn geobserveerd na SOM230 en OCT toediening. SOM230 250µg, echter, induceerde 1 uur na toediening een acute stijging van de bloedsuiker concentratie. Dit effect bleek niet verklaard door een absolute remming van de insuline spiegel door SOM230. Derhalve is geponeerd dat SOM230 mogelijk de werking van insuline negatief

beïnvloed, het induceert mogelijk insuline resistentie in perifere doelwit organen van insuline werking zoals de lever, skeletspieren en visceraal vet. Zoals beschreven in de **hoofdstukken III-3** en **III-4**, is de expressie van sst_1 en sst_2 mRNA in deze perifere doelwit organen van insuline werking een eerste vereiste voor een betrokkenheid van sst in de regulatie van insuline werking. De evaluatie van insuline receptor (IR) auto-phosphorylatie in de humane hepatoom cellijn Huh-7, welke endogeen sst_1 en sst_2 tot expressie brengt, heeft niet aangetoond dat SOM230 IR autophosphorylatie kan doen verlagen via mogelijke activatie van proteïne tyrosine phosphatasen. Andere oorzaken, bijvoorbeeld dephosphorylering van IRS eiwitten, welke down-stream van IR gelegen zijn, door SOM230 via sst_1 en/of sst_2 zullen nog opgehelderd moeten worden.

Met behulp van [^{125}I-Tyr^{11}]SRIF-14 radioligand binding en functionele cAMP respons studies in transiënt getransfecteerde HEK 293 cellen met verschillende expressie niveaus van sst_2/sst_5, wordt in **hoofdstuk IV-1** aanvullend bewijs aangeleverd dat de sst_2 een dominante rol heeft over sst_5 om adenylyl cyclase activiteit te remmen. Het verlagen van de intracellulaire cAMP concentratie via remming van het adenylyl cyclase is een van de belangrijke second messenger systemen welke betrokken zijn bij de suppressie van GH secretie. Onze fundamentele benadering om de functionele interactie te bestuderen tussen sst_2 en sst_5, was volledig in overeenstemming met de *in vitro* en *in vivo* correlatie tussen sst_2 mRNA expressie niveaus, maar niet met sst_5 mRNA, en de remmende werking van OCT en SOM230 op GH secretie door GH-producerende hypofyse adenomen. Experimenten met een sst_2-antagonist ondersteunden de klinische observatie van het voordeel dat SOM230 heeft, vergeleken met OCT, in die acromegalen geclassificeerd als partiële OCT-responder. In de aanwezigheid van de sst_2-antagonist was alleen SOM230 in staat om effectief adenylyl cyclase activiteit in sst_2 + sst_5 tot expressie brengende HEK 293 cellen te supprimeren. Kennelijk, als de sst_2 expressie laag was, kon OCT niet effectief werken via de sst_5 omdat OCT een 40-voudige lagere bindingsaffiniteit dan SOM230 heeft voor de sst_5. In overeenstemming met onze hypothese die gebaseerd op de klinische

observaties, ondersteunen de fundamentele experimenten het concept dat SOM230 van toegevoegde waarde kan zijn in de medicamenteuze behandeling van acromegalie, omdat deze multiligand effectief via sst_2 en sst_5 zowel GH secretie als adenylyl cyclase activiteit kan remmen. RNA interferentie is een interessante nieuwe methodologie waarmee het mogelijk moet zijn om een specifiek gen te 'silencen'. **Hoofdstuk IV-2** liet zien dat RNAi gen-constructen voor de sst_2 en sst_5 mogelijk in de toekomst bruikbaar kunnen zijn om de rol van de sst subtypen verder te bestuderen aangaande hun rol in de regulatie van hormoon (hyper-) secretie door sst-expresserende hypofyse adenoom cellen.

Hoofdstuk V beschrijft niet alleen het sst expressie patroon in hypofyse adenomen van patiënten met de ziekte van Cushing (CD), maar ook de functionele karakterisering van sst_2 en sst_5 receptoren in de regulatie van ACTH secretie. In patiënten die onbehandeld zijn voor CD, is gebleken dat OCT de ACTH en cortisol niet effectief kan onderdrukken, wat waarschijnlijk verklaard wordt door een down-regulatie van sst_2 expressie door glucocorticoïden. Deze hypothese wordt ondersteund door onze observaties in muizen corticotrophe AtT-20 cellen, waarin een down-regulatie van sst_2 mRNA en eiwit door glucocorticoïden is aangetoond. De supprimerende effecten op ACTH afgifte door AtT-20 cellen en primaire kweken van humane corticotrophe adenoom cellen gecombineerd met de observaties dat de sst_5 relatief resistent is voor down-regulatie door glucocorticoïden, suggereren dat de sst_5 een belangrijke rol speelt in de regulatie van ACTH secretie. Verder is gebleken dat voornamelijk de sst_5 tot expressie komt in humane corticotrophe adenoom cellen en sst_2 mRNA laag was in deze groep hypofyse adenomen. Dit ondersteunt nogmaals de klinische observatie dat OCT niet effectief is voor de behandeling van CD. Derhalve hypothetiseren wij dat SRIF-analogen met bindingsaffiniteit voor sst_5 en sst_2, of zelfs universele SRIF-analogen, mogelijkerwijs een nieuwe medicamenteuze behandelingsoptie kunnen worden om ACTH en cortisol hypersecretie in onbehandelde CD patiënten te controleren.

In **hoofdstuk VI-1** is een overzicht gegeven omtrent de rol van nieuwe

SRIF-analogen in de behandeling van neuro-endocriene tumoren. Een potentieel medisch probleem tijdens langdurige behandeling met de sst_2-prefererende SRIF-analogen OCT en Lanreotide is het ongevoelig worden van de tumor cellen voor de remmende werking van SRIF-analogen op hormoon secretie. Dit fenomeen wordt tachyphylaxie genoemd. Daarom hebben wij in **hoofdstuk VI-2** het optreden van tachyphylaxis voor de remmende werking van SRIF-analogen op adenylyl cyclase activiteit in sst_2 + sst_5 tot expressie brengende HEK 293 cellen verder onderzocht. Langdurige OCT-behandeling, in overeenstemming met klinische ervaring met OCT in carcinoid tumoren, resulteerde in een compleet ongevoelig worden van de sst_2 receptor. In tegenstelling tot OCT, induceerden SOM230 en BIM-23023, een sst_2-specifiek SRIF-analoog, een afname in gevoeligheid van de sst_2 welke geclassificeerd moet worden als zijnde een partiële tachyphylaxis. De sst_5, vergeleken met de sst_2, bleek in zijn geheel minder gevoelig te zijn voor het optreden van tachyphylaxis. Deze observaties met de sst_5 subtype wijzen wellicht op een mogelijke behandelingsoptie in patiënten met een neuro-endocriene tumor die ongevoelig zijn geworden voor de huidige generatie sst_2-prefererende SRIF-analogen. Tevens suggereren deze agonist-selectieve inducties van tachyphylaxis van een enkele sst subtype, dat aparte agonist-geïnduceerde receptor conformaties verantwoordelijk voor deze effecten kunnen zijn. Een potentieel nieuw medicamenteus gereedschap in de behandeling van neuro-endocriene tumoren inclusief hypofyse adenomen, BIM-23A760, bindend aan sst_2+sst_5 en de dopamine D2 receptor (D2R), is kritisch onderzocht in sst_2+sst_5+D2R tot expressie brengende HEK 293 cellen. Dit is beschreven in **hoofdstuk VI-3**. De functionele effectiviteit van BIM-23A760 in ons cellijn model ondersteunt niet het concept van oligo-heterodimerisatie tussen sst_2, sst_5 en de D2R. De effectiviteit van BIM-23A760, vergeleken met de mono-receptor SRIF-analogen en de D2R-agonist Cabergoline, in mono-receptor getransfecteerde HEK 293 cellen om adenylyl cyclase activiteit te remmen, bleek niet verschillend te zijn ten opzichte van hun effectiviteit in HEK 293 cellen die getransfecteerd waren met alle drie de receptoren. BIM-23A760, met eenzelfde bindingsaffiniteit voor D2R als zijn interne controle BIM-53097, bleek zelfs meer potent

dan BIM-53097 om adenylyl cyclase activiteit te remmen. Derhalve, hypothetiseren wij dat het dopastatine chimeer BIM-23A760 een specifieke ligand-monoreceptor interactie veroorzaakt ten opzichte van de individuele monoreceptor liganden, welke op zichzelf reeds een goede verklaring kan vormen waarom BIM-23A760 een betere potentie heeft.

In **hoofdstuk VII** worden zowel de resultaten van onze studies bediscussieerd, alsmede de mogelijke klinische significantie en potentiële toekomstige ontwikkelingen met het gebruik van nieuwe sst subtype selectieve en universele SRIF-analogen.

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List of publications
&
Curriculum Vitae

Publications included in the thesis:

1. **Hofland LJ, van der Hoek J, van Koetsveld PM, de Herder WW, Waaijers M, Sprij-Mooij D, Bruns C, Weckbecker G, Feelders R, van der Lely AJ, Beckers A, Lamberts SW** 2004 The novel somatostatin analog SOM230 is a potent inhibitor of hormone release by growth hormone- and prolactin-secreting pituitary adenomas in vitro. *J Clin Endocrinol Metab 89:1577-85*

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 12 september 1974 te Dordrecht. Na het Atheneum gevolgd te hebben op de openbare scholengemeenschap (O.S.G.) Walburg te Zwijndrecht, studeerde hij Biologie aan de Universiteit te Leiden. Het doctoraal examen werd behaald in 1997. Hierna werd aangevangen met de studie geneeskunde aan de Erasmus Universiteit te Rotterdam. Het artsexamen werd met lof afgelegd in 2002. Vervolgens werd in juni 2002 als arts-onderzoeker onder leiding van prof. dr. S.W.J. Lamberts en dr. L.J. Hofland aangevangen met het promotieonderzoek, zoals in dit proefschrift beschreven. Vanaf 1 september 2005 startte hij met de opleiding tot internist in het Albert Schweitzer Ziekenhuis te Dordrecht (opleider dr. A.C.M. van Vliet).

Gedurende zijn opleiding tot bioloog en arts, alsmede tijdens zijn promotieonderzoek, heeft de auteur tevens een sportieve volleybalcarrière genoten bij VC Nesselande te Rotterdam. In zijn acht jaar als semi-professionele volleyballer heeft hij onder andere drie Nationale indoor volleybal kampioenschappen, een Nationaal beachvolleybal kampioenschap, een zilveren medaille tijdens de Top Teams Cup Europa cup finale en een Nationale bekertitel gewonnen. Daarnaast heeft hij gedurende zijn eerste jaar als arts-onderzoeker deel uitgemaakt van het Nationaal volleybal team.