Somatostatin and dopamine receptors as molecular targets for the medical treatment of Cushing's disease

Christiaan de Bruin

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Somatostatin and dopamine receptors as molecular targets for the medical treatment of Cushing's disease

Somatostatine en dopamine receptoren als moleculaire doelwitten voor de medische behandeling van de ziekte van Cushing

Proefschrift

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Chapter 1

Introduction and aims of thesis

Based on:
C. de Bruin, R. A. Feelders, S. W. J. Lamberts and L. J. Hofland

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A. HARVEY W. CUSHING

Harvey Williams Cushing (1869-1939) was born as the 10th child of a well-educated, puritanical medical family in Cleveland (figure 1). He attended Yale University, graduated cum laude from Harvard Medical School and was trained as a general surgeon at Johns Hopkins under the famous but drug-addicted William Halsted. He proceeded to specialize in surgery of the brain and nervous system. In this way, he personally invented the field of neurosurgery and in the process of doing so, named a dozen of pathophysiological conditions after himself. Moreover, he introduced a number of ideas to the field of general clinical medicine, which are still of great value today: he was the first physician to use diagnostic X-rays on his own patients, he proposed the use of anaesthesia (Ether's) charts within the operating theatre after the avoidable death of one of his patients and also was the first doctor to use electrocoagulation during surgery (1).

Apart from his medical achievements, he was regarded as a World War I hero and furthermore a more than talented baseball and tennis player, a Pullitzer-Prize winning author, medical historian and artistic drawer. Personally, he was good friends with Sir William Osler and the Russian physiologist Pavlov and was a great fan of the 16th century Italian anatomist Andreas Vesalius. Unfortunately, Harvey Cushing was also a victim of the notorious 1918 Spanish flu, which left him with some chronic disability, not being

Figure 1: Harvey Cushing at work in the operating theatre
Source: www.dodd.cmcvellore.ac.in

able to walk more than a few steps at a time. Despite this disability, he proceeded to perform more than 2000 brain surgeries and wrote over 330 books and articles.

He was a hard-working, stern man who was both respected and feared by his residents and nursing staff for his sarcastic remarks and stormy outbursts. Not much of a family man to his wife and 5 children, he consistently placed his medical work ahead of anything else in life and could mourn for days after the death of a patient, blaming only himself. At the age of 70 he died in a way, which is both ironic as well as symbolic for the life he has lived. When working on a manuscript about Vesalius, he died of a myocardial infarction, presumably induced by the lifting of one of the heavy folio volumes of Vesalius' work. At autopsy, he had a posterior coronary artery occlusion, as well as a 1 cm colloid cyst of the third ventricle, in line with the common belief that doctors tend to develop the disease in which they have specialized.

B. CUSHING'S DISEASE: PATHOPHYSIOLOGY AND CURRENT TREATMENT

As early as 1932, Harvey Cushing reported the remarkable constellation of symptoms in a group of patients he had seen over the years, who showed pronounced abdominal and facial obesity, but also had remarkable wasting of the arm and leg musculature (2). In addition, they often had high blood pressure and could be severely depressed. Harvey Cushing was the first physician to discover that this full-blown clinical picture could be caused by a small basophilic adenoma in the human pituitary gland, secreting excessive amounts of ACTH. According to the common practice of that era, he named this new clinical entity after himself. In Cushing's disease (CD), the ACTH-producing pituitary adenoma stimulates the adrenal glands to produce excessive amounts of cortisol (figure 2). Chronic exposure to these high levels of endogenous cortisol has detrimental effects on a variety of organ systems including the cardiovascular, musculoskeletal and metabolic system (figure 3). Patients with CD are at substantial risk to suffer from any of the well-known sequelae of CD such as myocardial infarction, stroke, osteoporosis and depression (3). Age-adjusted mortality rates are significantly higher than those in the normal population (4).

As originally proposed by Harvey Cushing himself, the first line of treatment for this disease is the surgical removal of the ACTH-producing adenoma via a transsphenoidal approach. The introduction of transsphenoidal surgery instead of a classical craniotomy, which was customary at Harvey Cushing's time, single-handedly reduced intra-operative mortality during neurosurgery from 90% to below 10%. Since that time, many factors within and outside the operating theatre have improved, thereby gradually increasing

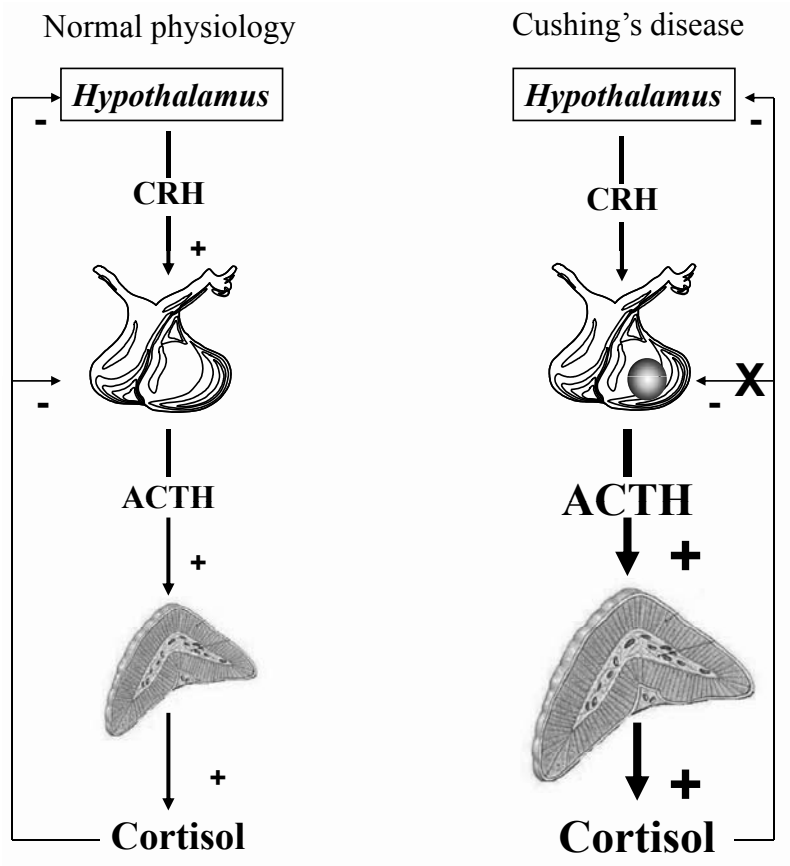

Figure 2: Normal physiology of the hypothalamus-pituitary-adrenal axis (left) and the pathophysiology of Cushing's disease (right). In the normal situation (left), hypothalamic CRH (corticotrophin releasing hormone) stimulates the anterior pituitary to secrete ACTH, which stimulates the adrenal glands to increase cortisol production. By a negative feedback system, increased levels of circulating cortisol decrease pituitary ACTH and hypothalamic CRH secretion. In Cushing's disease (right), a monoclonal expansion of ACTH-producing adenoma cells (grey circle) autonomously secretes excessive amounts of ACTH into the circulation. The excess ACTH stimulates the adrenal glands to produce high levels of endogenous cortisol. The pituitary corticotroph adenoma cells are partially resistant to negative feedback by circulating cortisol (black cross), leading to a state of sustained hypercortisolism.

the success rates for this type of surgery. Despite these great technical advances and further refinements of this surgical technique, selective transsphenoidal adenomectomy in patients with CD remains a highly challenging type of surgery to master. At present, even in most experienced hands, surgical cure rates decline to below 70% when patients are followed-up for at least 5 years after their initial surgery (5). A second transsphenoidal surgery in patients with a recurrence of CD is known to have significantly lower success rates than the primary surgery (6, 7). Other treatment options for patients with persistent or recurrent CD include conventional radiotherapy or gamma knife surgery.

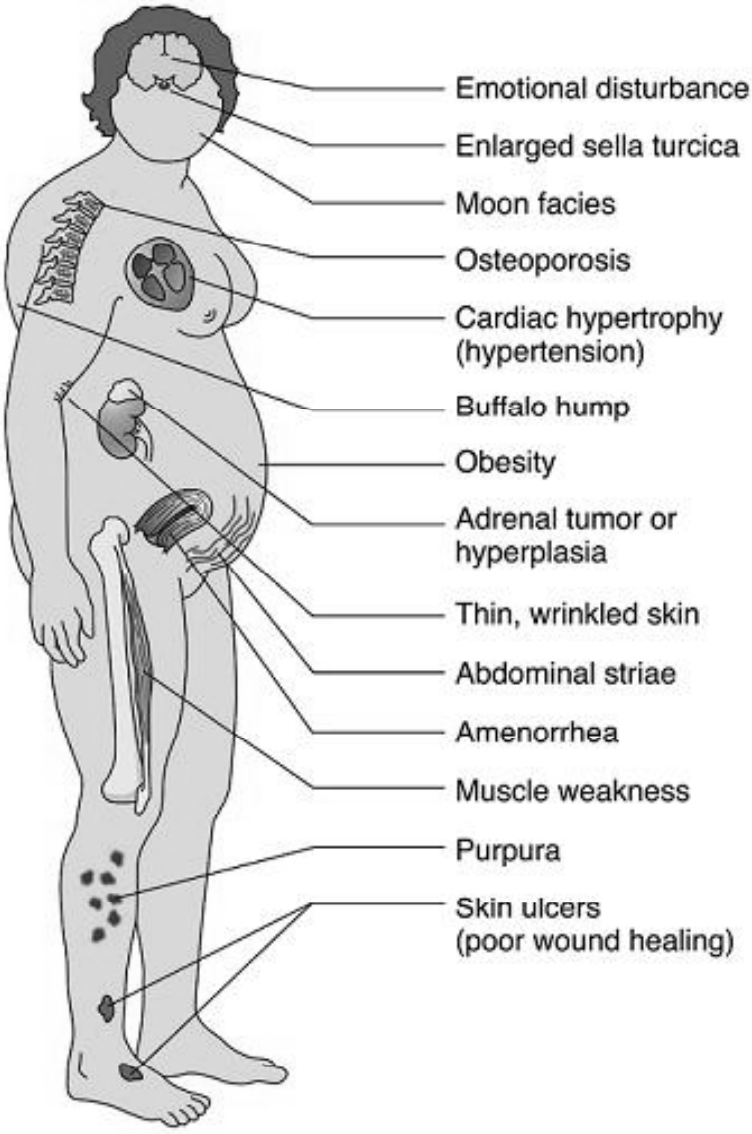

Figure 3: Clinical characteristics of patients with Cushing's disease. Source: Emanuel Rubin, john L. Farber, Pathology, 3rd edition, Lippincott-Raven Publishers (1999)

Both are effective at reducing ACTH hypersecretion in the majority of patients, but have a slow onset of action, with an average time until remission of 9-24 months (8). In addition, radiotherapy is accompanied by a significant risk of inducing secondary pituitary dysfunction, cranial nerve damage or secondary brain tumours (8-10). As a definitive cure, patients with persistent CD can undergo bilateral adrenalectomy, but this does have important implications in terms of lifelong dependence on hormone replacement therapy and the risk of future Addisonian crises, as well as Nelson's syndrome.

For the above reasons there is a clear rationale behind the search for an effective medical therapy for CD. A great number of drugs that act at the level of the pituitary, the adrenals or the glucocorticoid receptor itself, have been evaluated in the past decades with generally modest and variable results (11). Most compounds either show limited efficacy or are associated with serious toxicity and adverse events. For instance, the steroidogenic inhibitor ketoconazole has been shown to effectively decrease cortisol levels in about 50% of patients when used at high doses (12), but often causes considerable gastrointestinal side effects and carries a serious risk of medication-induced hepatitis, which limits its use as a long-term monotherapy in CD patients (13). Similarly, metyrapone can be effective in reducing cortisol levels in patients with CD, but can cause hypertension and hypokalemia. Blockade of the glucocorticoid receptor with mifepristone (RU-486) can improve symptoms of hypercortisolism, but the absence of a suitable biochemical parameter to monitor treatment efficacy makes dose titration difficult and this can result in severe adrenal insufficiency in some patients (14). In recent years, however, important new insights in corticotroph cell physiology and the receptors, which govern the regulation of ACTH release from these cells, have opened up the way for some potentially new medical therapies for CD.

C. RESEARCH ON NOVEL MEDICAL THERAPIES FOR CD

As the underlying pathophysiology of CD is located at the level of the pituitary corticotroph cell, it is plausible to direct any medical interventions primarily at this specific cell type. Different receptors have been identified within the human anterior pituitary, which may all be involved in the regulation of ACTH release and may therefore all represent potential targets for the development of new medical therapies. These include somatostatin and dopamine receptors, which will be the subject of this research thesis, but also the retinoic acid receptor, interferon gamma receptor and the peroxisome-proliferator activating receptor gamma. The potential of the latter receptors as novel therapeutic targets for the medical treatment of Cushing's disease are discussed in detail elsewhere (15)

One issue that negatively affects overall progress in the evaluation of all of these potential targets is the low incidence of human CD, approximately 1.2-2.4 cases/million/year (4, 16). This means that not only clinical studies are difficult to carry out because of low patient numbers, but also that *in vitro* research on primary corticotroph tissue from patients with CD is largely dependent on the scarce availability of these tissues after transsphenoidal surgery. It needs to be emphasized, that studies using these pri-

mary corticotroph adenomatous tissues, are crucial in the evaluation of the biological relevance of any novel therapeutic target receptor.

Interestingly, CD is a very frequent disorder in dogs with an estimated incidence of 1-2 cases/thousand/year (17, 18). In a few centers worldwide, including the Faculty of Veterinary Sciences of Utrecht University, the Netherlands, it is technically possible to perform transsphenoidal hypophysectomy in dogs with CD, resulting in favourable long-term outcome rates compared to medical therapy (19, 20). It also means that canine CD may provide a novel source of corticotroph adenoma tissue, which could facilitate *in vitro* research on the pathophysiology and treatment of CD in general. However, for a thorough evaluation of the feasibility of canine CD as a direct model for human CD, it is important to investigate the molecular make-up of these canine corticotroph adenomas, compared to their human counterparts.

AIM OF THESIS: To characterize canine corticotroph adenomas for the expression and functional role of two receptor families that are currently of interest as potential targets for the treatment of human CD, i.e. somatostatin and dopamine receptors, and to compare these results with the current knowledge on their expression in human corticotroph adenomas. In this way we aim to evaluate canine Cushing as a potential spontaneous animal model for human CD. The results of these studies are described in Chapter 2.

D. SOMATOSTATIN AND DOPAMINE RECEPTORS: GENERAL PHYSIOLOGY

Somatostatin and dopamine receptors

Somatostatin (SS) is a 14-or 28 amino acid-long cyclic peptide that is widely distributed throughout the human body. Its functions vary from increasing gastro-intestinal motility to neurotransmission within the central nervous system, mediating immune responses and inhibition of hormone release (21, 22). SS exerts its functions by binding to all five somatostatin receptor subtypes (sst_{1-5}), which belong to the family of G-protein coupled receptors (GPCRs) (23). Dopamine (DA) is a catecholamine with an equally wide range of functions including neurotransmission, control of vascular tone, renal function and hormone secretion (24). Also for DA receptors, five subtypes are known (D_{1-5}) that belong to the GPCR family, which are further classified into D_1-like (D_1, D_5) and D_2-like (D_2, D_3, D_4). D_1-like receptors generally mediate stimulatory functions, whereas most D_2-like receptors are associated with inhibition. Upon binding of SS or DA to their respective receptors expressed on the plasma membrane of target cells, multiple cellular effector systems can be activated, which include inhibition of Ca^{2+}-influx, inhibition of adenylyl

cyclase activity or stimulation of phosphotyrosine phosphatases, resulting in a variety of biological effects (figure 4) (23, 24). Both sst and DA receptors are abundantly expressed in the human neuro-endocrine system and in the tumours that are derived from it (25, 26). Most of the *in vivo* functions of SS and DA (D_2-like) receptors are inhibitory and, therefore, targeting these receptors with their natural agonists or synthetically derived analogs has provided opportunities for medical therapy of various neuro-endocrine disorders.

SS analogs and DA agonists

Soon after its discovery in 1972, SS was known to be a major regulator of GH release from the pituitary and was therefore of potential interest for the treatment of acromegaly (27). One of the characteristics of native SS, however, is its very short half-life in the circulation, which is approximately 3 minutes (28). For that reason, the production of synthetic SS analogs with a significantly longer half-life, was a major step forward in the treatment of this disorder. The first stable SS-analog produced was Octreotide (SMS 201-995), which has a half life of approximately 120 minutes after subcutaneous admin-

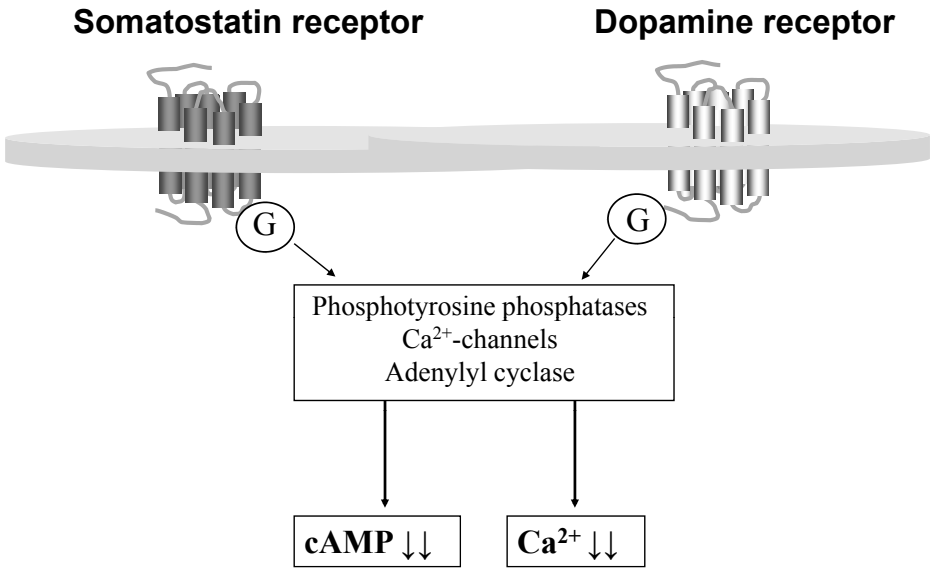

Figure 4: Schematic representation of somatostatin and dopamine receptors and their main second messengers. Both receptors are members of the family of 7-transmembrane spanning G-protein coupled receptors. Upon ligand binding, the activated Gi subunit of the receptors can result among others in activation of phosphotyrosine phosphatases and inhibition of Ca^{2+}-channels and the enzyme adenylylcyclase. The subsequent decreases in cAMP and intracellular Ca^{2+} can result in a variety of downstream cellular effects.

istration and was shown to reduce GH and IGF-1 levels in approximately two thirds of acromegalic patients (29, 30).

An important further step in the development of SS analogs was the discovery of the 5 somatostatin receptor subtypes in the 1990s. These findings clarified indirectly that the two available SS-analogs, Octreotide and its long-acting form Lanreotide, bind preferentially to the sst_2, but only modestly to sst_5 or any of the other subtypes. Native SS, on the other hand, binds with high affinity to all of its receptors (sst_{1-5}). In the subsequent years, evidence grew that not all neuro-endocrine tumours expressed receptor subtypes in a similar manner. Whereas growth-hormone producing adenomas generally expressed high levels of sst_2, other adenomas, such as corticotroph adenomas expressed considerably lower levels of sst_2 (figure 5). The concept that different neuro-endocrine tumours with important differences in sst expression profiles would require specific sst-targeting analogs, sparked the interest for the development of new types of SS-analogs that displayed high affinity for one or more SS receptor subtypes (table 1). One example of such a compound is BIM-23244, which is a bispecific SS-analog with high affinity for both sst_2 and sst_5. In GH-producing adenomas that were only partially responsive to Octreotide, this novel bispecific compound suppressed GH-production *in vitro* significantly more effective than Octreotide, probably through co-activation of sst_2 and sst_5 receptors (31). Another example is Pasireotide (SOM230), which is a multi-ligand SS-analog with high binding affinity for the sst_1, sst_2, sst_3 and sst_5 with IC_{50} values of 9.3, 1.0, 1.5 and 0.16 nM, respectively (table 1) (32). Its binding profile, which includes high sst_5-affinity, makes it a promising new drug in the treatment of a number of neuro-endocrine tumours, including CD (see below).

Dopamine agonists are an important class of drugs with a broad range of therapeutic indications, including neurological disorders (Parkinson's disease), cardiovascular dysfunction and neuro-endocrine disorders. They can be classified into either non-ergot (e.g. quinagolide) or ergot-derived (e.g. bromocriptine, cabergoline, pergolide). Bromocriptine has been known for many years to effectively inhibit prolactin (PRL) release in the majority of prolactinomas (33). With increasing knowledge on DA receptors, it also became evident that selectivity of dopaminergic compounds for DA receptor subtypes was of great importance for their overall efficacy and safety profile. In comparison with bromocriptine, cabergoline has a longer plasma half-life, binds with a higher affinity to the D_2 receptor, is better tolerated by patients and can induce normalization in patients with hyperprolactinemia that are proven to be resistant to bromocriptine therapy (34). The fulfilment of these criteria makes cabergoline a promising drug for the treatment of a number of neuro-endocrine disorders in which D_2 expression plays an imminent role.

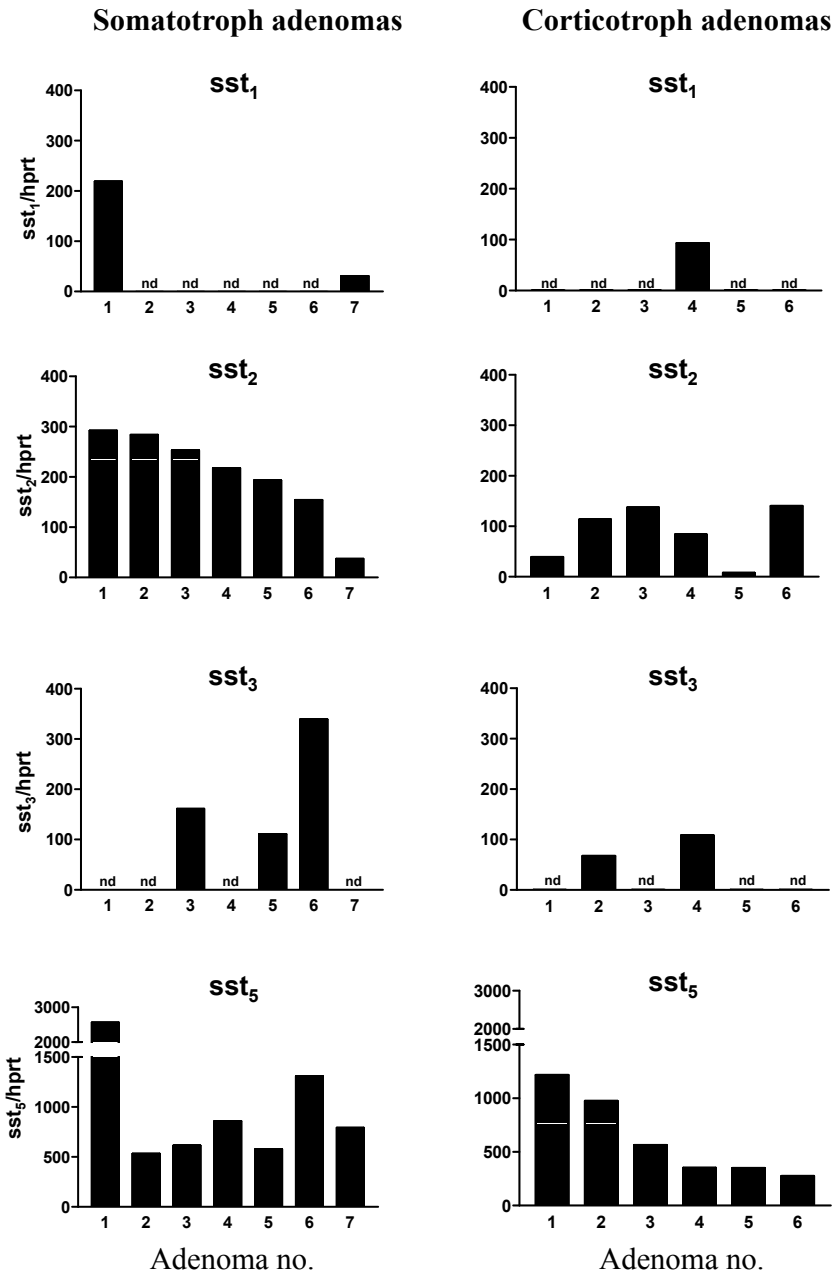

Figure 5: Overview of sst expression in 7 GH-producing (somatotroph) and 6 ACTH-producing (corticotroph) human pituitary adenomas. Note the difference in scale of the y-axis between sst_{1-3} and sst_5. Somatotroph adenomas have abundant expression of both sst_2 and sst_5 (left column), whereas corticotroph adenomas express sst_5 at similar levels but have a significantly lower expression of sst_2 (right column). Values are expressed as copy numbers relative to that of the reference gene hprt. N.d. = not detectable. Adapted from (57, 116).

The binding affinities of dopamine (DA), bromocriptine and cabergoline to the D_2 and D_4 receptors, are also shown in table 1.

Table 1 Binding affinities (IC_{50}) of SS-analogs and DA-agonists (in nM)

Compound	sst_1	sst_2	sst_3	sst_4	sst_5	D_{2Short}	D_{2Long}	D_4
SS-analogs								
Somatostatin (SS-14)	0.93	0.15	0.56	1.50	0.29			
Octreotide	280	0.38	7.10	>1000	6.30			
Pasireotide (SOM230)	9.3	1.0	1.5	>1000	0.16			
BIM-23244	>1000	0.3	133	>1000	0.7			
Dopamine agonists								
Dopamine						350	320	100
Bromocriptine						4.5	3.9	>1000
Cabergoline						0.53	0.41	81
Dopastatin chimera								
BIM-23A760	622	0.03	160	>1000	42		15*	

References: SS-binding data (32, 117), DA-binding data (118), BIM-analog binding data (31, 119)
* IC_{50} for D_2 receptor (both short and long isoform)

Chimeric somatostatin-dopamine compounds

The fact that many neuro-endocrine cells co-express both sst and DA receptors, has driven the hypothesis that these receptors may work synergistically. In 2000, Rocheville et al. published an important paper on the functional heterodimerization of sst_5 and D_2 receptors in stably transfected CHO-K1 cells, which resulted in overall enhanced biological potency (35). Based on these observations, new chimeric molecules have been synthesized that contain structural elements of both SS and DA compounds and therefore bind with high affinity to both sst and DA receptor subtypes. By binding to the two different receptors, these hybrid molecules were proposed to draw the receptors together in a spatial manner. This can lead to enhanced potency of the chimeric compound, compared to activation by two separate DA or SS analogs (36). At present it is not known, however, whether this enhanced potency is merely due to this proposed phenomenon of heterodimerization between the SS and DA receptors or whether other mechanisms may also be involved, such as superior activation at the level of individual receptors.

AIM OF THESIS: We aimed to further characterize the functional aspects of one of these new dopamine-somatostatin chimeric compounds, BIM-23A760, in vitro. The results of these studies are described in Chapter 7.

E. Somatostatin and dopamine receptors in Cushing's disease (CD)

1. Somatostatin analogs in Cushing's Disease

Sst expression in normal corticotroph cells

Whereas the role of hypothalamic SS as a principal regulator of pituitary GH-release has been firmly established (37), the effect of SS on ACTH release by the anterior pituitary gland has been less clear. Rat pituitary corticotrophs are known to express multiple sst, including sst_2 and sst_5 (38-40), but treatment of cultured rat corticotrophs with SS-14 does not result in inhibition of ACTH-release (41, 42). On the other hand, when rat pituitary cells are cultured in glucocorticoid-free media, SS-14 is able to decrease ACTH-release (43). In agreement with these findings, infusion of SS-14 or Octreotide does not alter ACTH-release in normal subjects (44-47), whereas both of these compounds can acutely decrease plasma ACTH levels in conditions of hypocortisolemia such as untreated Addison disease (48). These observations suggest that the presence of glucocorticoids reduces the inhibitory effects of native somatostatin and traditional SS analogs on ACTH release.

In vitro studies with SS-analogs in corticotroph cell lines and adenomas

The only available ACTH producing cell line from corticotroph origin is the murine AtT-20 cell line. A number of studies have indicated that in these cells sst_2 and sst_5 are principally involved in regulation of ACTH release and that selective agonists that target these subtypes effectively inhibit ACTH secretion (49-53). More recently it was found that especially sst_5 played a crucial role in regulating ACTH release in these cells and that sst_5-targeting agonists were more effective than sst_2-agonists in inhibiting ACTH release (54). Interestingly, pre-incubation with dexamethasone decreased the expression of sst_2 in these cells, but not of sst_5, and in line with these findings Octreotide, but not Pasireotide, lost most of its ACTH inhibiting potential after glucocorticoid pre-treatment (54). These data are in line with the original observations that glucocorticoids downregulate the total number of SS binding sites in cultured pituitary cells (55). Evidence for abrogation of sst_2-mediated effects by glucocorticoids has also been provided by Stalla et al (56). They found that Octreotide decreased ACTH levels in corticotroph adenomas *in vitro*, but not in CD patients *in vivo*. However, when these corticotroph adenoma cells *in vitro* were pre-treated with the glucocorticoid hydrocortisone, the ACTH inhibiting effects of Octreotide were abolished in one of the cultures. Given the generalized state of hyper-

cortisolism in CD patients and the relative resistance of sst_5 to glucocorticoid-induced down-regulation compared to sst_2, SS-analogs with high sst_5 affinity are of great interest in the development of new medical therapies for CD.

AIM OF THESIS: To investigate more in detail the effects of glucocorticoids on the expression levels of not only sst_2 and sst_5, but also D_2 in three human neuro-endocrine cell lines. These data could provide important insights into the biological processes that are responsible for the observed sst/DA receptor expression patterns in both pituitary-dependent and ectopic forms of Cushing's syndrome. In addition, these data may also provide further directions for the type and timing of future sst/DA directed therapies in these patients. The results of these studies are described in Chapter 3.

In 2005 and 2006, two studies were published that independently investigated sst expression in human corticotroph adenoma tissues, obtained at the time of transsphenoidal surgery. In the first study, Hofland et al. showed by quantitative PCR that sst_5 was highly expressed in 6/6 adenomas, whereas $sst_{1,2,3,4}$ were expressed at much lower levels (figure 5) (57). In concordance with this, functional studies in five additional adenomas demonstrated overall superior ACTH inhibition by Pasireotide (10nM) compared to Octreotide (10nM) after 72 hr.

In the second study, Batista et al. reported on a series of 13 corticotroph adenomas derived from both adult (n=7) and pediatric (n=6) CD patients (58). In this study, quantitative PCR demonstrated the expression of subtypes 1, 2, 4 and 5 in these adenomas, while at immunohistochemistry expression of all subtypes was found. Both of these methods showed the highest expression of the sst_5 subtype. Six of the adenomas were cultured *in vitro* and treated with Pasireotide. In 6/6 adenomas Pasireotide significantly decreased cellular proliferation rates (range 10-70%) as measured by uptake of fluorescent vital stain and in 5/6 a significant decrease in ACTH production was observed (range 23-56%) at doses of 1 to 10 nM after 48-96 hr. Furthermore, a dissociation was seen in some of the adenomas between the anti-secretory and anti-proliferative effects of Pasireotide, similarly to what has been described previously for GH-producing adenomas in response to SS-analog treatment (59).

AIM OF THESIS: Since the above data were derived from relatively small patient series, we aimed to investigate somatostatin receptor subtype expression in a larger set of human corticotroph adenomas. Furthermore, we concomitantly investigated dopamine receptor subtype expression in these adenomas to assess the degree of co-expression of both receptor subtypes. In this way, we aimed to get a better estimate of the percentage of patients with CD that could benefit from DA or SS receptor targeted therapy. The results of these studies are described in Chapter 4.

Clinical studies with SS-analogs in CD

Early studies showed that in patients with CD, Octreotide is not able to effectively reduce ACTH secretion and hence cortisol levels (56, 60, 61). In contrast, several smaller studies and case reports found that patients with Nelson Syndrome, i.e. an expanding ACTH-producing pituitary adenoma after bilateral adrenalectomy, did respond to Octreotide with reductions in ACTH (60, 62-64). This difference is readily explained by the differences in average circulating cortisol levels in both disease states and the effects of glucocorticoid-induced down-regulation of sst_2 receptors, as mentioned earlier (65).

Since then, few clinical studies have been reported that examined SS-analogue therapy in CD, until some important new insights developed. It was foremost the discovery that sst_5 was highly expressed in the majority of human corticotroph adenomas, which made the novel multi-ligand SS-analog Pasireotide an interesting compound to evaluate in patients with CD, due to its subnanomolar sst_5-affinity. A phase II multi-center clinical study has been performed in 29 patients with de novo or recurrent CD (66). Patients were treated with SOM230 600 µg twice daily over a 15-day period. Primary endpoint was normalization of 24-hour urinary free cortisol (UFC) levels. This study showed that out of 29 included patients, 5 (19%) obtained complete UFC normalization, while a total of 22 patients (76%) showed a decrease in UFC levels. Overall, Pasireotide was well tolerated in the 2 x 600 µg dose, except for some mild gastro-intestinal side effects such as nausea, abdominal pain and loose stools or diarrhoea. A major side effect of Pasireotide, however, which was already known from previous studies in acromegalic patients, was an overall increase in blood glucose levels. Overt deterioration of glucose tolerance was observed in approximately one third of the patients in this study.

2. Dopamine agonists in Cushing's Disease

DA receptor expression in normal corticotrophs

In humans, no firm data exist whether or not ACTH release is directly regulated by DA receptors in normal corticotroph cells. In rats it is known that the intermediate lobe in the pituitary is under tonic inhibitory control from dopaminergic neurons from the hypothalamus (67-69). The predominant cell type in the intermediate lobe is the melanotroph, which produces pro-opiomelanocortin (POMC). In the intermediate lobe POMC is processed into α-melanocyte stimulating hormone (α-MSH) and corticotropin-like intermediate lobe peptide (CLIP). This is different from the POMC-processing in anterior corticotroph cells, which mainly results in ACTH. The tonic inhibition by hypothalamic dopamine is thought to be exerted through D_2 receptors. This is demonstrated by the fact that D_2-deficient mice develop intermediate lobe hypertrophy with increased

POMC expression, elevated ACTH and corticosterone levels, resulting in adrenal gland hypertrophy (70). In humans, the intermediate lobe in the pituitary is a rudimentary structure, but is still thought to contain important biological functions. Human corticotroph adenomas arising from the intermediate lobe may have different characteristics than those arising from the anterior lobe, although some controversy exists around this subject (71, 72) .

In vitro studies with DA agonists in corticotroph cell lines and adenomas

Two reports have been published on the use of dopamine agonists in the murine corticotroph cell line AtT-20, but these have produced conflicting results. Farrell et al. found that treatment with the dopamine agonist bromocriptine did not reduce POMC mRNA expression in these cells, whereas Yin et al. did show that bromocriptine inhibited proliferation of these cells with induction of apoptosis (73, 74). The observed difference may be due to the fact that in the second study treatment with bromocriptine was significantly longer than in the first study (72 hr vs. 24 hr, respectively).

In 2004, Pivonello et al. investigated DA receptor expression in a series of 20 human corticotroph adenomas (75). They showed that the majority (80%) of these adenomas express the D_2 receptor as demonstrated by immunohistochemistry (IHC), receptor-ligand binding and RT-PCR. Of these D_2-positive adenomas, approximately 40% expressed the D_2 long isoform, 20% D_2 short and 40% expressed both isoforms. D_4 was expressed in 20% of cases, whereas D_1, D_3 and D_5 expression was not observed. Functional studies *in vitro* correlated very well with the D_2 expression data: adenomas high in D_2 expression responded well to either bromocriptine or cabergoline therapy with inhibition of ACTH release by 43 to 60%, whereas D_2-negative adenomas failed to respond. The D_2- expression data reported in this study are similar to those described by an earlier paper, where 11/16 (69%) of corticotroph adenomas, both functional and silent, expressed D_2 receptors as demonstrated by *in situ* hybridisation and immunohistochemistry (76).

Clinical studies with DA agonists in CD

The DA agonist bromocriptine has been widely evaluated for its potential use in human corticotroph adenomas. Overall, results of these studies have been variable. Although initial reductions in ACTH levels are evident in almost half of CD patients in response to bromocriptine administration, these reductions are often minor and sustained responses to bromocriptine therapy occur only in a small percentage of patients (77). Some studies have suggested that corticotroph adenomas arising from the intermediate lobe may be more likely to respond to bromocriptine (78).

Compared to bromocriptine, cabergoline binds with even higher specificity and affinity to D_2-receptors and has a longer duration of action (table 1) (34). Over the past decade, various case reports have demonstrated that ACTH-producing adenomas can be highly responsive to cabergoline therapy, both in patients with CD as well as in Nelson's Syndrome (table 2) (79-85). In some of these cases shrinkage of the corticotroph adenoma was observed on MRI (80-83). In the previously mentioned study by Pivonello et al., 20 patients with CD were treated with cabergoline at a dose of 1-3 mg/week for 3 months (75). This resulted in a significant decrease in urinary free cortisol (UFC) in 60% of patients and even complete UFC normalization in 40% of them. Interestingly, there was a very good correlation between the *in vitro* findings on D_2 receptor expression and the responses to cabergoline therapy *in vivo*. All D_2-expressing adenomas showed decreased cortisol levels *in vivo* in response to cabergoline therapy, whereas D_2-negative cases did not. Preliminary data from another research group showed similar *in vivo* response rates after cabergoline monotherapy (86). In this study, 10/29 CD patients (34%) had a complete normalization of 24 hr UFC after 3-6 months of therapy, while another 5 patients (17%) had a partial response (UFC ≤ 1.25 x ULN). In both studies, cabergoline therapy was generally well tolerated. Both research groups, however, also report the occurrence of (late) relapses in patients who were initially complete responders to cabergoline monotherapy. These treatment escapes reduce the long-term efficacy rates of cabergoline to approximately 27-40% (86, 87).

Table 2 Overview studies on cabergoline use in ACTH-producing pituitary adenomas (CD and Nelson)

Year	First author (ref)	No. of patients	Type	Macro-adenoma	Dose (mg/wk)	Duration (months)	Shrinkage observed	Remarks
1999	Pivonello (81)	1	Nelson	no	1-2	12	yes	normalization ACTH
2001	Petrossians (80)	1	Silent CD	yes	1.75	24	yes	restored cranial nerve function
2004	Miyoshi (82)	1	CD	yes	0.25-0.5	6	yes	decreased ACTH
2004	Casulari (83)	1	Nelson	yes	1.0	48	yes	normalization ACTH
2006	Shraga-Slutzky (85)	1	Nelson	yes	1.5-2	72	no	decreased ACTH (-90%)
2006	Illouz (79)	3	CD	no	1-3	1-9	no	normalization UFC in 2/3 patients
2007	Garcia (84)	1	Nelson	no	2	42	n.e.	decreased ACTH
2004	Pivonello (75)	20	CD	5/20	1-3	3	n.e.	40% full + 20% partial UFC response
2008	Godbout (86)	29	CD	n.m.	2	3-6	yes	34% full + 17% partial UFC response
2009	Pivonello (87)	20	CD	n.m.	1-7	3-24	n.e.	40% full remission after 2 yrs

n.e. = not evaluated

n.m. = not mentioned

One important issue that recently has dominated the field of medical therapy with DA-agonists has been the possible association between valvular heart disease and long-term therapy with the ergot-derived dopamine agonists (EDDA) pergolide and cabergoline. Two important papers were published in early 2007, which reported significantly increased risks (RR: 4.6-7.3) of valvular regurgitation in patients with idiopathic Parkinson's disease that had received chronic treatment with either one of these drugs (88, 89). Other studies have recently confirmed these data (90). The pathogenetic mechanism behind this deleterious side effect is thought to be the binding of EDDA to 5-HT2 receptors expressed in the endocardial tissue of heart valves (89).

These findings have led to a number of important actions, including the withdrawal of pergolide from the US market. The impact of these studies on the (future) use of cabergoline in patients with CD cannot be fully determined yet, as one important issue needs to be emphasized. The maximum dose of cabergoline prescribed in CD is around 0.65 mg per day (4.5 mg/week), whereas the patients with Parkinson's disease in the study by Zanettini et al. received an average daily dose of 3.6 mg/day (89). In the other study by Schade et al., an important risk difference was found between patients taking >3 mg cabergoline daily for more than 6 months (RR 50.3, 95% C.I: 6.6-381.4) compared to those who took less than 3 mg daily (RR 2.6; 95% C.I: 0.5-12.8) (88). Therefore, these observations in Parkinson's disease patients can not be directly extrapolated towards lower-dose cabergoline therapy in CD. Recent studies have shown that patients who are on long-term cabergoline therapy for prolactinoma, do not have an increased incidence of heart valve abnormalities, as assessed by cardiac echocardiography (91-95). Most important, perhaps, is the notion that mild tricuspid regurgitation is a common finding, which can be present in up to 40% of the normal population (96). Therefore, the clinical relevance of finding mild, echocardiographic valve abnormalities in patients on long-term cabergoline use remains unclear (96, 97). Nonetheless, until definitive conclusions can be drawn on this subject, most clinicians will agree that periodical evaluation of cardiac function in any patient on long-term cabergoline therapy, especially those who are on higher doses, should be performed during follow-up (98).

3. Combined treatment with SS analogues and DA agonists in CD

Due to the reported presence of both sst and DA receptors in human corticotroph adenomas and the fact that both receptor types can inhibit ACTH production *in vitro*, the concept of a combination therapy with both SS-analogs and DA-agonists in CD seems to be a feasible approach (99). These studies could be performed by co-treatment with individual SS-analogs and DA-agonists (pasireotide + cabergoline) or perhaps, in the near future, by administration of SS-DA-chimeric compounds such as BIM-23A760, which

displays high affinity for sst_2, D_2 and to a lesser extent sst_5 (see table 1). If functional heterodimerization of these receptor subtypes occurs *in vivo*, as has already been shown to occur *in vitro* by different groups, this type of treatment could result in greatly enhanced efficacy of these compounds (35). Also, as corticotroph adenomas can differ considerably in the total number of sst and D_2 receptors they express (57, 58, 75), targeting of multiple receptors could increase the overall response rate in this group as a whole, compared to the use of individual SS or DA agonists. This has already been shown for GH-producing adenomas, where BIM-23A760 had overall superior efficacy compared to individual sst_2, sst_5 or D_2-targeting agonists in terms of *in vitro* GH inhibition (100, 101). As it is known that also in CD only subsets of patients have responded to either cabergoline or pasireotide monotherapy *in vivo*, it may well be that similar phenomena occur in corticotroph adenomas and that combination therapy can increase overall response rates. Until now, no studies have been published that have investigated this hypothesis.

AIM OF THESIS: To investigate whether combined therapy with SS-analogues with sst_5 affinity and D_2 targeting agonists increases the percentage of CD patients that can be controlled biochemically during the standard pre-operative period of three months. We have investigated this in a multi-center clinical trial in patients with de novo or recurrent CD. Preliminary results of this ongoing study are presented in Chapter 5.

Theoretically, co-treatment with sst and DA agonists may have other advantages as well. As stated before, the inefficacy of sst_2-preferring compounds in CD, is probably due to down-regulation of sst_2-expression by high levels of circulating glucocorticoids (54, 56, 57). Inversely, if combined treatment with these analogs is effective and thus lowers cortisol levels in these patients, this could result in re-expression of sst_2. The latter would result in enhanced efficacy of SS-analogs with sst_2-affinity and hence strongly increase pharmacotherapeutical options in these patients (65, 102).

F. SOMATOSTATIN AND DOPAMINE RECEPTORS IN ECTOPIC ACTH-PRODUCING SYNDROME (EAS)

Ectopic ACTH-producing Syndrome (EAS)

Some peripheral neuro-endocrine tumours can secrete excessive amounts of ACTH, which can lead to the clinical entity known as the Ectopic ACTH-producing Syndrome (= Ectopic Cushing's Syndrome). Primary therapy is preferably the surgical removal of the neuro-endocrine tumour, but in some cases this is not technically possible. Bilateral adrenalectomy can offer a definitive cure, but has the same disadvantages as described

earlier for patients with persistent Cushing's disease who undergo this procedure. For that reason, medical therapy could be an important secondary treatment option in selected cases of EAS.

Somatostatin analogs in EAS

For some decades it has been known that neuro-endocrine tumours that cause Ectopic ACTH-producing Syndrome (EAS), such as bronchial carcinoids or small cell lung cancer (SCLC), often express functional SS receptors. A number of smaller studies and case reports have been published on the use of Octreotide in patients with EAS. Interestingly, Octreotide was efficacious in lowering cortisol levels in a significant number of these patients, as opposed to the studies performed in patients with CD (103-106). This discrepancy is further confirmed by the fact that many patients with EAS have positive lesions on ^{111}In-pentreotide scan (OctreoScan), whereas most patients with CD do not (107). The observation that many of the EAS producing neuro-endocrine tumours have functional sst_2 receptors, despite the chronic hypercortisolism they are exposed to, could be explained by aberrant glucocorticoid receptor signalling in these tumour cells. This has been investigated extensively by a number of research groups over the past twenty years. It was found that many of the cell lines, derived from EAS producing small-cell lung carcinomas, carry gross mutations in the genetic sequence of the glucocorticoid receptor (GR) (108, 109). These can be located either in the DNA-binding or the ligand-binding domain, but can also involve a number of transcription factors. The loss of function of the GR has important impact on POMC production in these cells. Any form of negative feedback is generally lost in these cells, leading to excessive and uninhibited production of POMC and ultimately, the full clinical spectrum of Cushing's Syndrome. Another result of aberrant GR functioning, may be that glucocorticoid-induced down-regulation of somatostatin receptor subtype 2 (sst_2) does not occur in these tumours, as opposed to pituitary-derived corticotroph adenomas. This could well explain the relatively high degree of positive OctreoScans and reported efficacy of Octreotide in this group of neuro-endocrine tumours (110, 111). One main concern with the use of SS analogs in EAS, however, appears to be the long-term control of hypercortisolism. Although initial responses to Octreotide are frequent, these are not always sustained and treatment escapes are commonly encountered, due to a number of possible mechanisms of tachyphylaxis (112).

AIM OF THESIS: To illustrate the clinical relevance of glucocorticoid-induced changes in somatostatin receptor subtype expression in EAS, we have described our in vitro and in vivo findings on sst and DA receptor expression patterns in a patient with EAS, before and after glucocorticoid-lowering therapy. The results of these studies are described in Chapter 6.

Dopamine agonists in EAS

Farrell et al. showed in 1992 that the dopamine agonist bromocriptine could effectively inhibit POMC mRNA and ACTH precursor secretion in a small cell lung cancer cell line (CORL103), that is known to cause EAS (73). After these initial observations, to our knowledge no (clinical) studies have been performed that investigated the potential use of DA compounds in EAS, until a recent study by Pivonello et al. (113). In this study, 6 patients with EAS-causing carcinoid tumours (4 lung, 1 thymic, 1 pancreatic) underwent surgery. Five out of these 6 resected EAS tumours expressed D_2, as determined by IHC. Three patients had persistent EAS after surgery and were treated with cabergoline at 3.5 mg/week for 6 months. All three patients had measurable D_2 mRNA and 2 out of 3 had D_4 mRNA expression on RT-PCR. Two patients had complete normalization of UFC after 3 months of cabergoline treatment, although one of them had a treatment escape afterwards. Of note, the long-term responder had the strongest overall D_2 expression, including the D_2 short isoform, and was also D_4-receptor positive. In other pituitary tumours this expression profile has been associated with a good response to cabergoline therapy (75, 114). Despite the small size of this study, it is probable that at least a subgroup of EAS patients could benefit from D_2-targeted treatment, but obviously these results need to be confirmed in larger series.

Combined treatment with SS analogues and DA agonists in EAS

A recent case report suggests the potential synergism between sst and DA receptors in EAS. In this case, a man with EAS due to a lung carcinoid tumour was treated medically after incomplete surgical removal. Cortisol levels normalized only temporarily with either a SS-analogue (Lanreotide) or a dopamine agonist (Cabergoline) alone. However, when both drugs were given simultaneously, based on co-expression of sst_5 and D_2 that was demonstrated by RT-PCR on the resected tumour specimen, the patient came into complete and prolonged remission (115).

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Chapter 2

Expression and functional analysis of dopamine receptor subtype 2 and somatostatin receptor subtypes in canine Cushing's disease

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ABSTRACT

Cushing's disease (CD) is a severe disorder characterized by chronic hypercortisolism due to an ACTH-secreting pituitary adenoma. Transsphenoidal adenomectomy is the treatment of choice in humans with CD but recurrences occur frequently. Finding an effective and safe medical treatment for CD may improve long-term clinical outcome. The recent demonstration of expression of somatostatin receptor subtypes (mainly sst_5) and dopamine D_2 receptors in human corticotroph adenomas offers the possibility for medical treatment of CD with novel somatostatin analogues and dopamine agonists. Investigation of the effects of these drugs is hampered by the low incidence of CD in humans. Interestingly, CD is a frequent disorder in dogs with striking clinical similarities with CD in humans. Therefore, we investigated the expression and functional role of D_2 and sst receptors in corticotroph adenoma cells from 13 dogs with active CD that underwent therapeutic hypophysectomy and normal anterior pituitary (NAP) cells from 5 dogs. Quantitative RT-PCR and immunohistochemistry revealed that both in CD and NAP, sst_2 was the predominant receptor subtype expressed, whereas D_2 was modestly expressed and sst_5 was expressed only at very low levels. In primary cultures of canine adenomas (n=7), the sst_2-preferring agonist octreotide also showed the strongest ACTH-suppressive effects. In conclusion, canine corticotroph adenomas provide an interesting model to study CD, but differences in sst and dopamine receptor expression between humans and dogs should be taken into account when using dogs with CD as a model to evaluate efficacy of novel somatostatin analogues and dopamine agonists for human CD.

INTRODUCTION

Cushing's disease (CD) is a severe endocrinological disorder due to an ACTH-producing pituitary adenoma. The resulting chronic hypercortisolism causes significant morbidity and, if left untreated, mortality in these patients (1). Primary treatment of CD is transsphenoidal selective adenomectomy (2), but only results in long-term cure in 50-80% of patients (3). Secondary treatments such as radiotherapy or bilateral adrenalectomy are generally effective, but can cause permanent hypopituitarism or the necessity of life-long adrenal hormone replacement therapy, respectively.

For that reason, finding an effective and safe medical therapy for human CD can be of great importance for those CD patients that are not cured by neurosurgery alone. Various drugs have been used in patients with CD, but most of them have not been efficacious in long-term treatment or are associated with an unfavorable safety profile (4). Novel drug targets have been identified, however, as it was found that the somatostatin receptor subtype 5 (sst_5) and the dopamine (DA) receptor subtype 2 (D_2) are expressed in the majority of human corticotroph adenomas (5-7). Compounds that target these receptor subtypes, such as the multiligand somatostatin analogue with high sst_5-affinity pasireotide (SOM230) and the D_2-agonist cabergoline, have already shown in some *in vitro* and *in vivo* studies to decrease ACTH release by corticotroph adenoma cells and thus lower cortisol levels (5, 6, 8).

For the development of new medical therapies in human CD, research on primary corticotroph adenoma tissue is crucial. The efficacy of new compounds in CD can only be genuinely tested in the cell type they are primarily directed at, i.e., the human corticotroph cell. This tissue can only be obtained at the time of transsphenoidal adenomectomy in a CD patient. Due to the low incidence of CD of approximately 1.2 to 2.4 cases/million/year (9, 10) and the fact that 80-90% of these cases are due to microadenomas with a diameter of less than 10 mm (11, 12), there is a severe shortage of human corticotroph tissue, which limits research options in human CD. For that reason, finding ways to increase the availability of primary corticotroph adenoma tissue is a major challenge in this research field.

In contrast to the situation in humans, CD is a frequent endocrinological disorder in dogs, with an estimated incidence of 1 to 2 cases/1000/yr (13-16). Canine CD, also referred to as pituitary-dependent hyperadrenocorticism (PDH), has a remarkably similar pathophysiology and clinical presentation as CD in humans and can hence be regarded as a spontaneous animal model for human CD (17). In dogs with CD true microadenomas are rare and pituitaries are frequently enlarged (18). Medical treatment of dogs with CD

involves the use of adrenolytic drugs such as mitotane or an inhibitor of steroidogenesis such as trilostane (19, 20). In the Netherlands, hypophysectomy has been performed in dogs with CD since 1993 and has proven to be a safe and effective treatment (18, 21, 22). The procedure consists of a complete hypophysectomy via a transsphenoidal approach as is described in detail elsewhere (22).

Given the high incidence of CD in dogs, the high degree of similarity with human CD and the availability of corticotroph adenoma tissue obtained at hypophysectomy, we hypothesized that evaluation of the efficacy of new compounds for treatment of human CD may be tested first in canine corticotroph adenoma tissue. Therefore, our main study aim was to characterize these canine corticotroph adenomas for the expression and functional role of those receptor subtypes that are of primary interest in the research of human CD: somatostatin receptor subtype 2 (sst_2), somatostatin receptor subtype 5 (sst_5) and dopamine receptor subtype 2 (D_2), and to compare these results with the current knowledge on human corticotroph adenomas.

MATERIALS AND METHODS

Study population

Thirteen dogs (5 females (4 spayed) and 8 males (3 castrated)) with Cushing's disease (i.e. pituitary-dependent hypercortisolism, PDH) from various breeds were included in the study (Table 1). The median age was 8 years (range 5-14 years) and the median body weight was 23.2 kg (range 6.7-48.0 kg). Hypercortisolism was diagnosed by clinical signs, routine laboratory investigation and determination of the urinary corticoid-to-creatinine ratio (UCCR) in two consecutive morning urine samples as described previously (23-26). The mean UCCR was 116.7×10^{-6} (range $26.5\text{-}302.5 \times 10^{-6}$; normal $<10 \times 10^{-6}$) (18). After collection of the second urine sample, three oral doses of 0.1 mg dexamethasone per kg body weight were administered at 8 h intervals and the next morning a third urine sample was collected (high dose dexamethasone suppression test). In 10 dogs the UCCR in the third sample was less than 50% of the mean in the first 2 samples and PDH was diagnosed (18). In 2 cases with less than 50% suppression, dexamethasone-resistant PDH was confirmed by measurements of plasma ACTH concentrations and further supported by visualization of the adrenals by ultrasonography and pituitary imaging (27-30). Computed tomography (CT) of the pituitary gland revealed pituitary enlargement in each case, except one (C8), with a mean pituitary height-to-brain area ratio (P/B) of 0.58 (range 0.30-1.00; pituitary enlarged when $P/B > 0.31 \times 10^{-2}\ mm^{-1}$) (31). Plasma cortisol, ACTH and α-MSH concentrations were determined with assays that have been

described previously (32). Pre-operative mean (+range) plasma values were: α-MSH 27.8 (<5-224) pg/ml, cortisol 196.9 (61-414) nmol/l and ACTH 21.5 (9.3-41.8) pmol/l (see Table 1 for reference values). Microsurgical transsphenoidal hypophysectomy was performed as published previously (22).

Unaffected pituitary tissue was obtained from five Beagle dogs, which had been euthanized for reasons unrelated to the present study and for which approval was obtained from the Ethical Committee of Utrecht University, the Netherlands. The pituitary gland was collected within 10 minutes after euthanasia. The anterior pituitary was separated from the neurointermediate lobe and the anterior pituitary was processed for analysis.

Surgical tissue and cell isolation

During transsphenoidal hypophysectomy, pituitary adenomatous tissue was identified macroscopically by the veterinary surgeon and resected. A representative part of the adenoma was fixated in 4% buffered paraformaldehyde and sent for histopathology for haematoxylin and eosin (HE) staining and immunohistochemistry to evaluate ACTH, α-MSH and GH expression (33). The surplus adenomatous tissue was immediately placed in a pre-chilled (4 ˚C) solution of Minimal Essential Medium (MEM) with Earle's salts, supplemented with 10% fetal calf serum (FCS), L-glutamine (2 mmol/l), penicillin (10^5 U/l) and fungizone (0.25 mg/l). Media and supplements were obtained from Invitrogen (Breda, the Netherlands). Upon arrival in the laboratory, the adenoma tissue was further divided into two parts: one part was snap-frozen on dry ice and stored at –80˚C for qPCR studies; the other part was kept overnight at 4˚C in MEM. Next day, the latter adenoma part was washed in HBSS/HSA, dispersed with dispase 10^3 U/l (Roche, Almere, the Netherlands) + collagenase 2 mg/ml (Sigma Aldrich, Zwijndrecht, the Netherlands) at 37ºC for 1 h and resuspended in MEM complete culture medium. Viable pituitary cells were counted in a standard haematocytometer.

Cell distribution and culture

The average yield per tumor in terms of viable canine pituitary cells was 2.4 x 10^6 cells (range: 0.5-11.0 x 10^6). Of these cells, 0.2 x 10^6 were used for qPCR studies and 0.1 x 10^6 for the preparation of cytospins for immunohistochemistry (IHC, see below). The remainder of the cells was cultured in 48 well plates (Corning, Cambridge, USA) at a density of 10,000 cells/well for 4-6 days at 37 ˚C in a humidified incubator in 5% CO_2. At that time, culture media were refreshed and incubations were started with the different DA agonists and SS analogues for 4-72 h. Both basal and corticotropin-releasing hormone (CRH)-induced ACTH-release were studied. At the end of the incubation period, media

Table 1. Clinical characteristics of canine patients included in this study

Case[1]	Breed	Gender[2]	Age (yr)	Body weight (kg)	Pit size[3] (mm)	P/B[4]	UCCR[5] (x 10^{-6})	DEX[6] (%)	ACTH[7] (pmol/l)	αMSH[8] (pg/ml)	Cort[9] (nmol/l)	Rem[10]	Histopath diagnosis[11]	Immunohistochemistry
N1	Beagle	F	2	10.4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	normal	n.a.
N2	Beagle	F	2	9.2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	normal	n.a.
N3	Beagle	F	2	12.0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	normal	n.a.
N4	Beagle	F	2	8.4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	normal	n.a.
N5	Beagle	M	5	10.8	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	normal	n.a.
C1	Sib. Husky	M	10.6	29.0	5-7-5	0.34	33.5	96.1	38.4	15.0	121	yes	Adenoma	ACTH+, αMSH+, GH-
C2	Dachshund	FC	8.4	7.7	5-5-4	0.35	26.5	86.4	17.5	6.5	61	yes	Adenoma	ACTH+, αMSH+, GH-
C3	Boxer	FC	5	32	9-9-8	0.42	241.0	96.8	12.2	16.5	265	yes	Adenoma	ACTH+, αMSH+, GH-
C4	Golden retriever	FC	10	28.4	10-11-11	0.56	31.0	-12.9	19.9	10.0	93	yes	Adenoma	ACTH+, αMSH+, GH-
C5	Vizla	MC	14	21.0	15-21-18	0.98	n.a.	n.a.	9.3	19.5	86.5	yes	Adenoma	ACTH+, αMSH+, GH-
C6	Stabyhoun	M	11	24.0	7-9-8	0.45	55.3	63.7	10.3	224	146.5	yes	Adenohypophysis	ACTH+, αMSH+, GH+
C7	Bernese M. Dog	F	6.7	48.0	12-13-12	0.69	100.0	61.0	41.8	5	319	n.a.	Adenoma	ACTH+, αMSH+/-, GH-
C8	Petit Bas. Gr.	MC	7.8	13.9	5-6-5	0.30	302.5	33.2	14.8	<5	414	yes	Adenohypophysis	ACTH+/-, αMSH-, GH+
C9	Labrador retriever	FC	8	36.5	12-12-12	0.56	30.7	88.3	22.2	20.5	192	yes	Adenoma PI[13]	ACTH+. αMSH+/-, GH-
C10	Beagle	M	8	23.2	17-15-16	1.00	74.5	75.8	30.4	20	172	yes	Adenoma	ACTH-, αMSH-, GH-
C11	Mongrel	M	6.5	12.8	15-14-16	0.99	81	60.5	20.8	7	231.5	no[12]	Adenoma	ACTH+/-, αMSH-, GH-
C12	Dachshund	M	11	6.7	8-10-10	0.55	207	76.1	21.0	<5	180.5	yes	Adenoma	ACTH-, αMSH-, GH-
C13	Beagle	MC	5.6	16.3	7-9-10	0.38	217.5	80.2	20.8	12.5	277.5	yes	Adenoma	ACTH+, αMSH+, GH-

[1] = cases: N1-N5: normal dogs; C1-C13: patients with Cushing's disease
[2] = gender: F = female intact, FC = female castrated, M = male intact, MC = male castrated
[3] = pituitary size (in mm) as measured on pre-operative helical CT (height - width - length)
[4] = pituitary height-to-brain area ratio x 10^{-2} mm^{-1} (P/B ratio ≤ 0.31 = normal sized pituitary, P/B ratio > 0.31 = enlarged pituitary)
[5] = pre-operative urinary corticoid-to-creatinine ratio (UCCR) (ref < 10 x 10^{-6}); values are the mean of two morning urine samples with 1 day interval
[6] = pre-operative degree of UCCR suppression after high-dose dexamethasone: 100 = complete suppression, 0 = no suppression of UCCR
[7] = pre-operative plasma ACTH (ref 1.1-18.7 pmol/l); values are the mean of two samples with an interval of 10-15 min
[8] = pre-operative plasma α-MSH (ref <36 pg/ml); values are the mean of two samples with an interval of 10-15 min
[9] = pre-operative plasma cortisol (ref 11-136 nmol/l); values are the mean of two samples with an interval of 10-15 min
[10] = patient post-operative in remission at time of writing, i.e., UCCR < 5 x 10^{-6} (yes/no)
[11] = diagnosis as stated by veterinary pathologist based on haematoxylin & eosin staining and immunohistochemistry for ACTH, α-MSH, and GH
[12] = recurrence at 4 months after hypophysectomy, following initial remission
[13] = adenoma of pars intermedia (PI)
n.a. = not available

were collected and stored at –80˚C for hormone analysis after addition of aprotinin (4 x 10^5 IU/ml medium; Bayer, Mijdrecht, the Netherlands) to prevent ACTH degradation. All experimental conditions were performed in quadruplicates.

Hormone analysis in vitro

ACTH production by the corticotroph cells *in vitro* was measured using a commercially available, non-isotopic, automatic, chemiluminescence immunoassay system (DPC Immulite, Los Angeles, USA). Intra- and interassay coefficients of variation were 5.6 and 7.8% respectively.

Design of canine sst_2, sst_5 and D_2 primers

The sequences of the canine housekeeping gene hypoxanthine phosphoribosyltransferase (hprt), sst_2, sst_5 and D_2 genes are available at the NCBI website (www.ncbi.nlm.nih.gov) with the following accession numbers: AY283372 (hprt); AY702068 (sst_2); XM_547202 (sst_5); NM_001003110 (D_2). Primers and probes were designed with Primer Express® software (Applied Biosystems, Foster City, USA) and ordered from Sigma Aldrich (Zwijndrecht, the Netherlands). Their sequences are depicted in Table 2.

Table 2. Primer-probe sequences

Primer/probe	Sequence 5'-3'	Bases
sst_2 forward	GCCATACTATGACCTGACCAGCA	23
sst_2 reverse	TGTTGCCACACAATCCAATGA	21
sst_2 probe	FAM-TGCAGTCCTCACATTCATATATTTTGTGGTCTGC-TAMRA	34
sst_5 forward	TGCTGGTCATCTGCCTCTGTT	21
sst_5 reverse	GCCGGACGCCTTCACC	16
sst_5 probe	FAM-CCTGCTCATCGTGGTC-TAMRA	16
D_2 forward	TGGCCACGCTCGTCATG	17
D_2 reverse	TGAATTTCCACTCACCTACCACC	23
D_2 probe	FAM-CCTGGGTTGTCTACCTG-TAMRA	17
hprt forward	GCTTGCTGGTGAAAAGGACC	20
hprt reverse	GAATTTCAAATCCAACAAGTCAGGT	25
hprt probe	FAM-CTCGAAGTGTTGGCTATA-TAMRA	18

Quantitative PCR

Expression analysis by quantitative PCR (qPCR) was performed both on the 2 x 10^5 cells obtained via the isolation procedure, as well as on a representative part of adenoma tissue that had been stored at –80˚C directly post-operatively. For qPCR we used a previously

described method (34). In short, poly(A^+) mRNA was isolated from the corticotroph cells with the use of Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway). The poly (A^+) mRNA was eluted in H_2O (65˚C) for 2 x 2 minutes and used for cDNA synthesis in a Tris buffer [50 mM Tris-HCl (pH 8.3), 100 mM KCl, 4 mM DTT and 10 mM $MgCl_2$] with 10 units RNase inhibitor, 2 units avian myeloblastosis virus Super Reverse Transcriptase and 1 mM of each deoxynucleotide triphosphate in a final volume of 40 µL. This was incubated for 1 h at 42˚C and the resulting cDNA was diluted 5-fold in 160 µl sterile H_20. One twentieth of the total cDNA library was used for quantification of hprt, sst_2, sst_5, and D_2 mRNA levels. The total reaction volume (25 µl) consisted of 10 µl cDNA and 15 µl TaqMan Universal PCR Mastermix (Alphen a/d Rijn, the Netherlands). Primers and probes were used at final concentrations of 300nM (both primers) and 200 nM (probe). Real-time quantitative PCR was performed in 96-well optical plates with the TaqMan Gold nuclease assay (Applied Biosystems, Roche, USA) and the ABI Prism 7700 Sequence Detection System (Perkin Elmer, Foster City, USA). After two initial heating steps at 50˚C (2') and 95˚C (10'), samples were subjected to 40 cycles of denaturation at 95˚C (15 sec) and annealing at 60˚C (60 sec). All samples were assayed in duplicate. Values were normalized against the expression of the housekeeping gene hprt. Dilution curves were constructed to calculate PCR efficiencies (E) for every primer-probe set (35). Efficiencies were: sst_2 2.01, sst_5 1.77, D_2 1.96 and hprt 1.84. Estimated copy numbers were calculated using the comparative threshold method with efficiency correction, as described previously (36). To exclude genomic DNA contamination in the RNA, the cDNA reactions were also performed without reverse transcriptase and amplified with each primer pair. To exclude contamination of the PCR reaction mixtures, the reactions were also performed in the absence of cDNA template, in parallel with cDNA samples.

Assessing purity of corticotroph cell population

Three steps were taken to secure the purity of the examined corticotroph adenoma tissue. First of all, the veterinary surgeon provided us only with pituitary tissue that was macroscopically adenomatous. When the surgeon assessed the pituitary tissue to be a mix of adenomatous and unaffected tissue, this was specifically noted. Secondly, a part of the isolated cells (1.0×10^5) was used to check for ACTH-immunopositivity on freshly prepared cytospins (see below for methods). Only isolated cell populations with significant ACTH-immunopositivity were eligible for analysis. As a third and final step, the expression of growth hormone (GH) and pro-opiomelanocortin (POMC) mRNA was analyzed in all samples with Bio-Rad My-IQ detection system (IQ SYBR green Supermix and My-IQ Bio-Rad, Veenendaal, the Netherlands) with final primer concentrations of 400 nM according to previously published protocols (37). For GH and POMC, the ribosomal protein S19 (rps-19) was used as a reference gene (38). Ratios of GH/POMC mRNA

expression were established in normal anterior pituitary cells (N1-5) and compared to those in the corticotroph adenoma samples (C1-13).

Neuro-D1 expression

In order to investigate the possible origin of the corticotroph adenoma (anterior vs. intermediate lobe), we also assessed Neurogenic Differentiation factor D1 (Neuro D1) mRNA expression in all samples, using the same qPCR protocol as for GH and POMC and with rps-19 as reference gene. Neuro-D1 is a transcription factor that promotes POMC expression and is a corticotroph marker in mice, dogs and humans (39, 40). It is highly expressed in the normal canine anterior lobe but not in the intermediate lobe (41).

Dexamethasone and sst_2 mRNA expression

To study the effects of glucocorticoids on sst_2 expression, isolated corticotroph cells were plated at a density of 100,000 cells/well and cultured for 72 h in the presence or absence of the glucocorticoid dexamethasone (DEX) 10 nM, the glucocorticoid receptor antagonist RU-486 100 nM or their combination. After 72 h, cells were lysed and mRNA expression levels of sst_2 and hprt were determined. All experimental conditions were performed in quadruplicates.

Immunohistochemistry (IHC): paraffin-embedded tissue and cytospins

The expression of ACTH and sst_2 was assessed in representative adenoma tissue by means of IHC according to a previously published method (42). Formalin-fixed, paraffin-embedded corticotroph adenoma tissues were cut (5µm), deparaffinized, rehydrated, heated in citrate buffer (pH 6.0) for 20' at 100˚C for antigen retrieval and incubated with the following primary antibodies: anti-ACTH (Santa Cruz, mouse monoclonal, 1:100, 1 h RT) and anti-sst_2 (Gramsch, rabbit polyclonal, 1:2000, o/n 4˚C). This was followed by 30' incubation at RT with Poly-AP-Goat anti Mouse/Rabbit IgG from PowerVision+ (ImmunoVision Technologies Co, Brisbane, CA, USA) and 30' incubation in New Fuchsin solution. Slides were counterstained with HE and cover slipped. Negative controls included omission of the primary antibody and preabsorption with an immunizing receptor peptide (100 nM) for the sst_2 polyclonal antibody. Three different commercially available antibodies against the human D_2 and two against the human sst_5 receptor were tested on canine normal anterior pituitary tissues and on a number of canine corticotroph adenomas. Unfortunately, none of these antibodies resulted in specific immunohistochemical staining.

To check for corticotroph purity of the adenoma specimen obtained at surgery (see above), cytospins of freshly isolated adenoma cells were made using a Cytospin 4 machine (Thermo Shendon Limited, Astmoor, U.K.), in which 2 x 10^4 cells were spun onto adhesive microscopic slides (Starfrost, Braunschweig, Germany). Subsequently they were air-dried, fixed in acetone for 10' and next, a similar IHC protocol as described above was used with an anti-ACTH antibody dilution of 1:600. In these cytospins, we counted the percentage of ACTH-positive cells as a measure of the percentage of corticotrophs in our isolated cell population.

Test substances

Test substances were obtained from Novartis Pharma AG, Basel, Switzerland (octreotide, pasireotide), Sigma Aldrich (RU-486), Pharmacia, Milan, Italy (cabergoline) and the Erasmus MC pharmacy (dexamethasone, CRH).

Statistical analyses

All data were analyzed with GraphPad Prism software (San Diego, CA, USA). Data on hormone release are expressed as mean ± SEM. All experiments were run in quadruplicate. Overall differences between treatment groups were determined by ANOVA. In case of significant differences found by ANOVA, a multiple comparison between groups was performed with a Newman-Keuls test. Correlation analyses were performed between the expression levels of NeuroD1, sst or D_2 receptor subtypes and/or corresponding preoperative hormone levels by determining Spearman's correlation coefficients. P-values less than 0.05 were considered statistically significant.

RESULTS

Study population follow-up

Remission of hypercortisolism occurred in 12 of the 13 dogs and was confirmed by resolution of clinical signs and UCCR values < 5 x 10^{-6} within 8 weeks after hypophysectomy. In one dog (C11) hypercortisolism recurred 4 months post-operatively. One other dog was lost to follow-up (C7). Histopathology revealed pituitary adenoma in 11 of 13 cases, with an adenoma originating from the pars intermedia in one case (C9). Immunostaining was positive for ACTH in 11 of 13 cases (Table 1).

Purity of obtained corticotroph tissue

Macroscopically pure adenoma tissue was identified by the surgeon in 9 of 13 cases. In the remaining cases the resected tissue was a mixture of adenoma and unaffected (pre-existent) pituitary tissue. Cytospins that were prepared from the isolated corticotroph cells, showed variable but significant ACTH-immunoreactivity in all cases that were analyzed (Table 3).

GH and POMC mRNA expression was determined in the 5 normal anterior pituitaries (NAP) and in the 13 adenomas (Table 3). The mean (± SEM) POMC/GH ratio in the 5 NAP cases was 0.36 ± 0.18. We defined pure corticotroph adenomas as having a POMC/GH

Table 3. mRNA expression data and immunohistochemistry cytospins

Case[1]	Tissue[2]	ACTH+[3]	POMC/GH[4]	Classification[5]	NeuroD1[6]
N1	N	n.a.	0.35	normal	0.22
N2	N	n.a.	0.10	normal	0.16
N3	N	n.a.	0.07	normal	0.08
N4	N	n.a.	0.22	normal	0.21
N5	N	n.a.	1.05	normal	0.23
C1	C	n.a.	231495.07	pure adenoma	0.04
C2	C/N	3+	3.58	non-pure	0.43
C3	C/N	1+	0.02	non-pure	0.10
C4	C	4+	5920.75	pure adenoma	2.27
C5	C	3+	149.43	pure adenoma	0.93
C6	C	3+	11.45	pure adenoma	0.29
C7	C/N	3+	0.17	non-pure	0.63
C8	C/N	n.a.	0.03	non-pure	0.09
C9	C	4+	548.86	pure adenoma	0.06
C10	C	n.a.	12.82	pure adenoma	0.17
C11	C	3+	856.95	pure adenoma	9.67
C12	C	1+	6230.95	pure adenoma	4.43
C13	C	3+	1.82	non-pure	0.50

[1] = N1-N5: normal dogs; C1-C13: patients with Cushing's disease

[2] = Macroscopic appearance of resected tissue as judged by veterinary surgeon. C: pure adenoma tissue; C/N: mixture of adenoma and unaffected tissue

[3] = Percentage ACTH-positive cells on cytospin: 1+ (0-10%); 2+ (10-20%); 3+ (20-30%); 4+ (>30%); n.a. = not available

[4] = POMC/GH mRNA ratio in normal anterior pituitary cells (N1-N5) and in corticotroph adenoma cells (C1-C13)

[5] = Classification of tissue: normal (= unaffected anterior pituitary tissue); pure adenoma tissue; non-pure (mixed adenoma-unaffected) tissue

[6] = NeuroD1/rps-19 mRNA (x 10^{-2}) expression

mRNA ratio of ≥10 x higher than the POMC/GH mRNA ratio observed in NAP. In this way, 8/13 adenomas were classified as pure adenomas and 5/13 adenomas as a mixture of adenoma and unaffected (= non-pure) pituitary tissue. Four of the latter 5 adenomas had been classified macroscopically by the surgeon as being a mixture. One case (C13) was assessed by the surgeon as pure adenoma but the POMC/GH mRNA ratio *in vitro* was low, indicating non-pure pituitary tissue.

mRNA expression: sst, D_2 and NeuroD1

In the corticotroph adenoma cells, which were obtained after cell dispersion *in vitro*, there was a strong but highly variable expression of the sst_2 receptor subtype (median 1.90; range 0.22-26.28) with two adenomas (C1, C6) showing very high sst_2 expression levels (Figure 1). D_2 was moderately expressed (median 0.75; range 0.00-8.07) and sst_5 was expressed at very low levels (median 0.02; range 0.00-0.49). These results were confirmed in similar but independent experiments with RNA that was extracted from the primary adenoma tissue that had been stored directly post-operatively at –80˚C. In these experiments a similar mRNA expression pattern was observed (data not shown). For comparison, expression levels in the normal anterior pituitaries were (median and range): sst_2 (7.98; 3.81-18.7), sst_5 (0.30; 0.08-0.66) and D_2 (0.96; 0.45-2.98). The anterior pituitary marker NeuroD1 was variably expressed among the adenomas with a median value of 0.43 x 10^{-2} (range 0.04-9.67 x 10^{-2}), which was higher than that of NAP (median 0.21 x 10^{-2}, range 0.08-0.23 x 10^{-2}, Table 3). No significant correlations were found be-

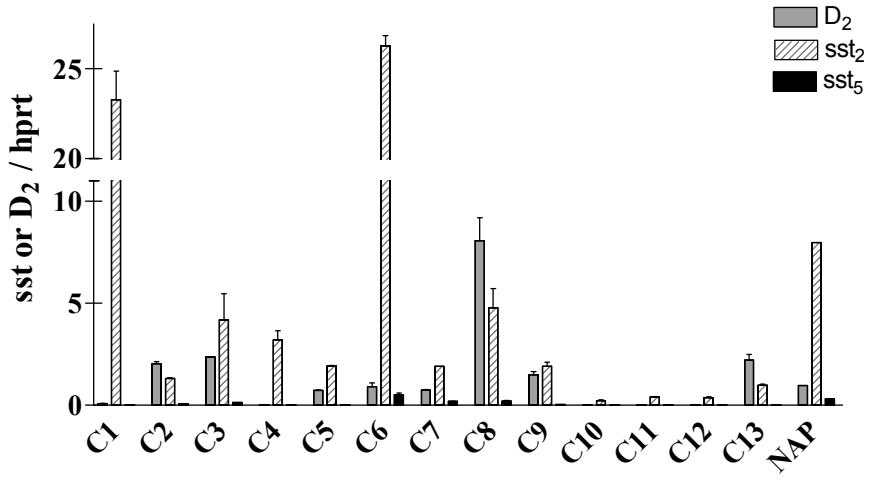

Figure 1: Overview of sst_2, sst_5 and D_2 mRNA expression in canine corticotroph pituitary adenomas (C1-13) and in normal anterior pituitary tissue (NAP). All expression levels are normalized against the housekeeping gene hprt. Values for C1-C13 represent the mean of two duplicate measurements ± SEM. For comparison, the median expression level in NAP (n=5) is depicted.

tween NeuroD1 and sst-D_2 receptor subtype expression or with pre-operative hormone levels (Spearman's correlation coefficients: $p>0.05$).

In vitro culture data

For seven pure corticotroph adenomas, we were able to measure the effects of DA/SS analogues on ACTH inhibition *in vitro*. Mean basal ACTH production in these adenomas was 86 pmol/l at 4 h (range 33-188), 222 pmol/l at 24 h (range 56-471) and 591 pmol/l at 72 h (range 88-1240). Stimulation with CRH 10 nM induced a mean 2.0 fold increase (range 0.7-3.9) in ACTH production at 4 h compared to basal. In all adenomas combined, the sst_2-preferring agent octreotide (OCT) was most effective at inhibiting 4 h CRH-induced ACTH release (-27%, $p<0.01$ vs. control), whereas the multiligand SS-analogue pasireotide (PAS) (-18%, $p<0.05$) and the D_2-agonist cabergoline (CAB) (-13%, $p<0.05$) were less effective (Figure 2A). All compounds were used at the 10 nM concentration. Combining CAB with either OCT or PAS did not increase ACTH inhibition compared to OCT or PAS alone (OCT+CAB -23%, $p<0.05$ vs. control; PAS+CAB –20%, $p<0.05$). Of note, the two adenomas with the highest sst_2 mRNA expression (C1, C6) were also most responsive to OCT (10 nM) treatment in terms of 4 h CRH-induced ACTH inhibition: C1: OCT -67%, $p<0.001$ (Figure 2B); C6: OCT -74%, $p<0.001$ (Figure 2C). The other 5 adenomas (C4, C5, C9, C11, C12) showed minor to moderate (10-30%) ACTH inhibition in response to the different compounds.

Parallel to this, we investigated ACTH inhibition in these adenomas without CRH stimulation. At the 24 h time point a similar pattern of response to DA and SS analogues was observed. Data for all adenomas combined were: OCT -20% ($p<0.001$ vs. control), PAS -13% ($p<0.05$) and CAB -9% ($p>0.05$). In these experiments without CRH stimulation, adding CAB to OCT or PAS increased the overall ACTH inhibition: OCT+CAB: -24% ($p<0.001$ vs. control) and PAS+CAB: -20% ($p<0.001$) (Figure 2D). Similar patterns of inhibition were observed after 72 h, although average levels of ACTH inhibition were lower at this time point (data not shown).

Immunohistochemistry (IHC)

In normal canine pituitary tissue, sst_2 was expressed in the anterior pituitary, but immunoreactivity for sst_2 was especially strong in cells of the intermediate lobe (Figure 3). The staining pattern was primarily cytoplasmatic and absent with omission of the primary antibody or when co-incubated with an immunizing peptide. In a subset of patients (n=5), we were able to perform IHC for sst_2 on the corticotroph adenoma tissue that was formalin-fixed and paraffin-embedded directly after surgery. For these adenomas, the

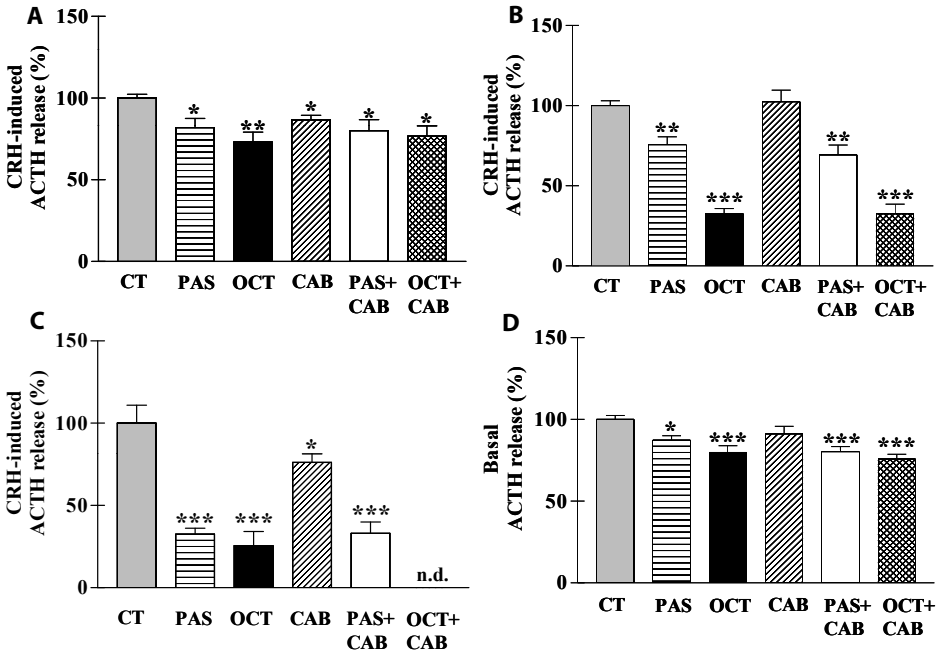

Figure 2A-D: ACTH inhibition in canine corticotroph adenomas. Fig 2A-C: Primary corticotroph cells were cultured and stimulated for 4 hours with CRH 10 nM in the presence or absence of pasireotide (PAS) 10 nM, octreotide (OCT) 10 nM, cabergoline (CAB) 10 nM or their combination. Data are shown for all adenomas combined (fig 2A), adenoma C1 (fig 2B) and adenoma C6 (fig 2C). Fig 2D: Basal ACTH production after 24 h in the presence or absence of the same compounds as above (data for all adenomas combined). After 4 h or 24 h, respectively, media were collected and ACTH levels were determined. All experimental conditions were performed in quadruplicates. Values represent percent change ± SEM relative to control (CT). Control was CRH alone (for CRH-data) or untreated cells (for basal data). * = p-value <0.05; ** = p<0.01; *** = p<0.001 vs. control; n.d. = not determined.

results of IHC for sst_2 expression corresponded well with the previously described mRNA data. In one of the tumors with a very high sst_2 mRNA expression (C1), a strong overall sst_2 staining was observed with clear co-localization of sst_2 and ACTH-immunoreactivity (Figure 4), whereas the other corticotroph adenomas showed staining of minor intensity (C4, C5) or only of isolated cells (C2, C3). Due to unavailability of canine-specific antibodies, we were not able to test for sst_5 or D_2-immunopositivity in these tissues.

Dexamethasone and sst_2 mRNA expression

To explore potential regulation of receptor subtype expression by glucocorticoids, we investigated the effects of the synthetic glucocorticoid dexamethasone (DEX) on sst_2 mRNA expression in two primary corticotroph cultures (C4, C12) with a sufficiently high cell yield that allowed us to perform additional experiments. Treatment with DEX 10 nM for 72 h caused increased sst_2 mRNA expression in both adenomas with an average

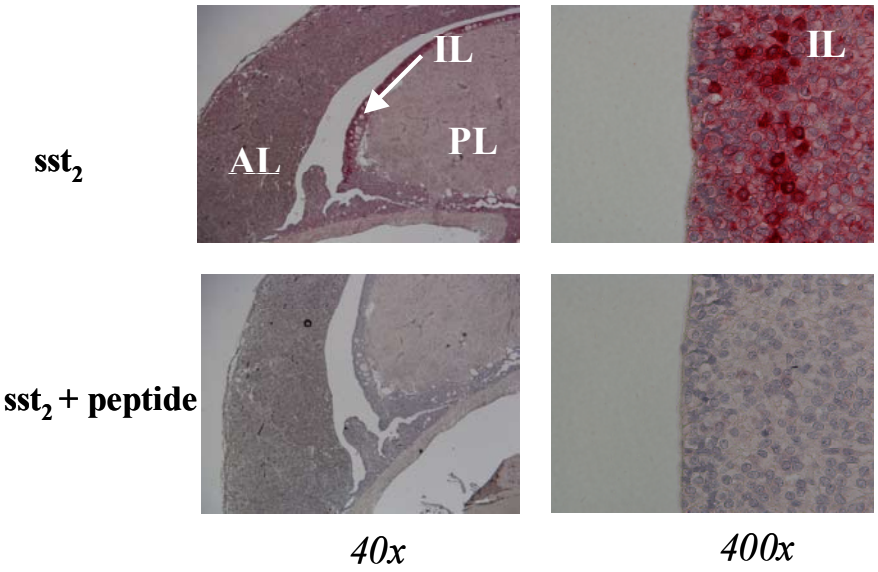

Figure 3: Immunohistochemistry for sst_2 expression in the normal canine anterior pituitary. Top panel left, *magnification 40x*: strong sst_2 expression in the anterior lobe (AL) and the intermediate lobe (IL, arrow), but not in the posterior lobe (PL). Top panel right, *400x*: cytoplasmatic staining for sst_2 in individual cells of the intermediate lobe. Bottom panel left *(40x)* and right *(400x)*: no staining in negative control with immunizing receptor peptide.

increase of 61% ($p<0.05$ vs. control, Figure 5), with C4: +51%, $p>0.05$ and C12: +71%, $p<0.05$. Addition of the glucocorticoid antagonist RU-486 100 nM abolished these effects. The effects of DEX could not be investigated for sst_5 and D_2, as the expression levels of these subtypes were too low in these particular adenomas.

DISCUSSION

Canine corticotroph adenomas resected during transsphenoidal surgery constitute a new and interesting source for retrieving considerable amounts of valuable primary corticotroph tissue. This primary tissue can be of great value for research regarding pituitary developmental processes, as well as etiology, diagnosis and therapy of pituitary disorders (43). Due to the high incidence of CD in dogs, surgical specimens of fresh adenoma tissue become available at a routine basis and have a high average yield in terms of viable corticotroph adenoma cells. Furthermore, these cells remain viable in culture, produce ACTH in significant amounts, are CRH-responsive to a variable degree and can respond to commonly used agonists *in vitro*. The fulfilment of all of these criteria makes canine corticotroph adenomas a feasible and readily used model for the study of (human) Cushing's disease.

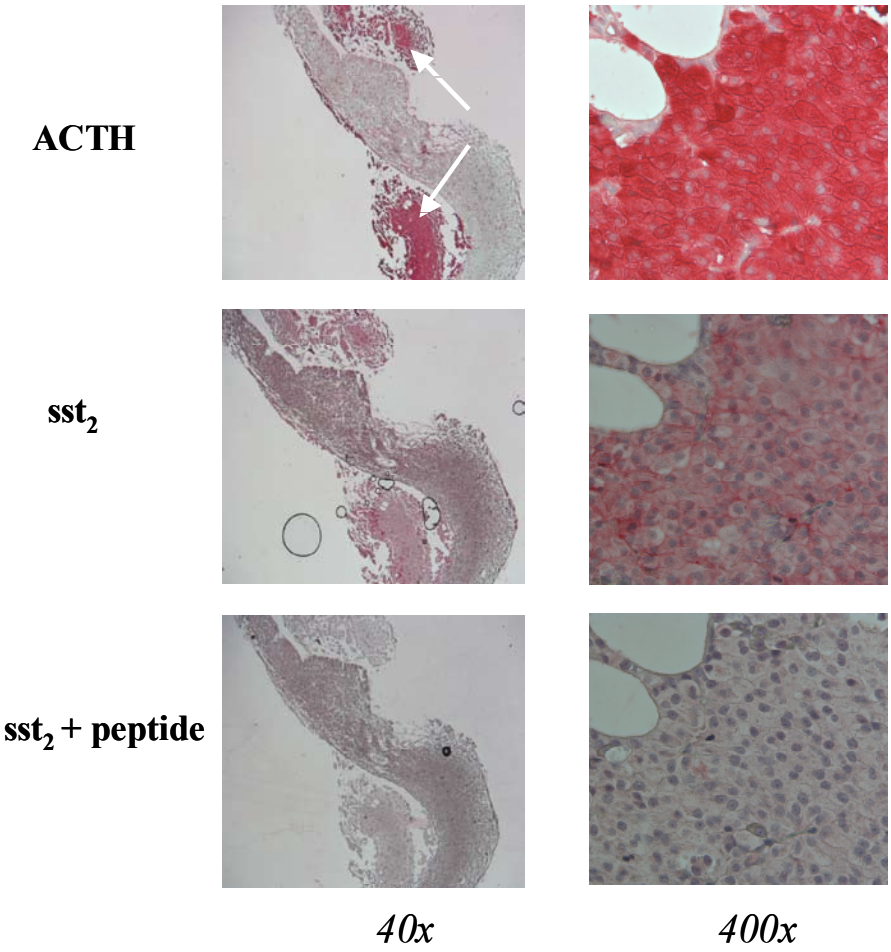

Figure 4: Immunohistochemistry for sst_2 expression in canine corticotroph adenoma C1. Top panel: strong ACTH expression in the adenomatous tissue (arrow), magnification left *40x* and right *400x*. Middle panel: sst_2 expression is evident in the areas of ACTH-positive adenoma tissue, *40x* and *400x*. Bottom panel: no staining in negative control with immunizing receptor peptide, *40x* and *400x*.

The main objective of our present study was to evaluate the expression and functional significance of dopamine (D_2) and somatostatin receptor subtypes (sst_2 and sst_5) within these canine corticotroph adenomas. These receptor subtypes are the main focus of much of the current research into human CD and agonists that target these receptor subtypes have already been used in clinical studies with promising results (6, 8). From this perspective, canine corticotroph adenoma tissue could constitute a useful tool to further explore efficacy and mechanism of action of novel SS or DA compounds for future use in human CD.

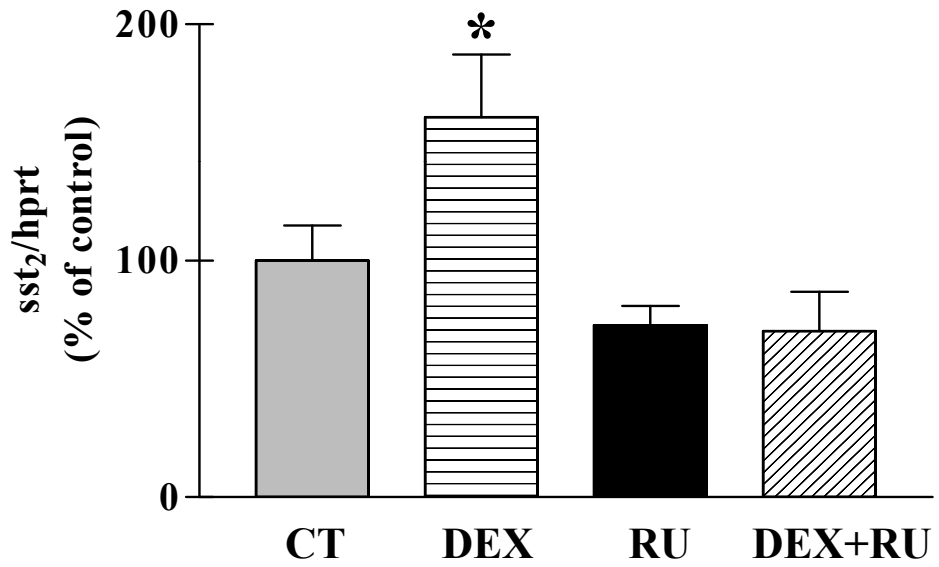

Figure 5 Glucocorticoid regulation of sst2 mRNA expression. Adenoma cells of C4 and C12 were cultured in the absence or presence of the glucocorticoid dexamethasone (DEX) 10 nM and/or the glucocorticoid receptor antagonist RU-486 100 nM. After 72 h, cells were lysed and mRNA expression levels of sst2 and housekeeping gene hprt were determined by qPCR. Hprt expression levels did not vary among treatment groups. All experimental conditions were performed in quadruplicates. Values represent percent change ± SEM relative to control. * = p-value <0.05 vs. control (CT).

Despite the many striking similarities in etiology and clinical presentation between human and canine CD, canine corticotroph adenomas differ clearly from their human counterparts in terms of SS and DA receptor expression patterns. Canine corticotroph adenomas mainly express sst_2, whereas D_2 and especially sst_5 are expressed at much lower levels. The predominance of sst_2 is observed at the mRNA level, as demonstrated by quantitative PCR, and confirmed at the protein level by immunohistochemical studies. In agreement with this, the sst_2-preferring agonist octreotide is the most efficacious agent in inhibiting ACTH release in both basal and CRH-stimulated conditions, whereas the multiligand SS-analogue pasireotide is significantly less effective. The lower efficacy of pasireotide compared to octreotide is readily explained by its 2.5 fold lower binding affinity for the sst_2 receptor (IC_{50} 1.0 vs. 0.38 nM, respectively) (44) in combination with the low overall expression of sst_5 in canine corticotroph adenomas. The D_2 agonist cabergoline shows some efficacy in the 7 cultured adenomas combined, albeit lower than octreotide and pasireotide. This finding is in line with the lower D_2 mRNA expression compared to sst_2 observed in this study.

Nonetheless, this modest level of D_2 receptor expression could still prove to be of functional value. In a recent study by Castillo et al. dogs with CD were treated with cabergo-

line (0.07 mg/kg/wk) for 1 year, which resulted in an overall response rate of 42.5% (45). One factor that could explain this observed difference between the *in vitro* and clinical efficacy of cabergoline could be the duration of treatment. It is known from studies in human patients with CD that it can take up to 3 months before the maximal cortisol-inhibiting effects of cabergoline are observed (6). In this respect, our *in vitro* data on ACTH inhibition after 4-72 h may not necessarily reflect the full potential of cabergoline as a drug in canine CD. On the other hand, the high levels of sst_2 expression both on the mRNA and the protein level, in combination with the superior efficacy of octreotide in cultured canine corticotroph adenomas, suggest an even stronger role of this receptor subtype as a therapeutic target. Based on our findings, a clinical study to investigate the effects of an sst_2-preferring compound such as octreotide on ACTH and cortisol levels in canine CD could be of great interest to see if superior response rates could be achieved with the use of such compounds, compared to those obtained with cabergoline. In addition to this, it would be very interesting to study whether combined targeting of sst_2 and D_2 receptors, either by co-treatment with the individual SS/DA analogs or by the use of novel chimeric SS-DA molecules could result in even higher clinical efficacy.

To return to our original research question, the receptor expression pattern observed in canine adenomas is remarkably different from the one observed in human corticotroph adenomas, where sst_5 and D_2 are the predominant receptor subtypes and sst_2 expression is generally low. The reasons for this dissimilarity between canine and human corticotroph adenomas are yet unknown. One important factor, however, appears to be the difference in regulation by glucocorticoids of receptor subtype expression. Down-regulation of sst_2 expression by glucocorticoids has been demonstrated in murine corticotroph AtT-20 tumor cells and is also thought to explain the low sst_2 expression in human corticotroph adenomas (5, 46, 47). Striking, therefore, was the observation in our study that this glucocorticoid-induced down-regulation did not occur in canine corticotroph adenomas. In fact, treatment of the canine corticotroph cells *in vitro* with dexamethasone increased the expression of the sst_2 receptor, as was observed in two different adenomas. From a future perspective, it would be interesting to see whether these differences can be ascribed to the 7% inhomology between the canine and the human sst_2 genetic sequence, as it is possible that this genomic variation is also present in areas within the human sst_2 gene that are known to contain glucocorticoid-responsive elements.

It is important to emphasize that sst and D_2 are not the only receptors that have been linked to regulation of ACTH secretion in corticotroph cells. Receptors such as the retinoic acid receptor (RAR) and peroxisome-proliferator-activated receptor-γ (PPARγ) have also been shown to decrease ACTH regulation in different *in vitro* and rodent models and have therefore been implicated as potential new targets for medical therapy of CD in humans

(48, 49). Most notably, retinoic acid was used in a recent clinical study in dogs with CD and showed significant clinical efficacy (50). In this respect, it would be very interesting to evaluate canine corticotroph adenomas for the presence and distribution of novel drug targets such as RAR and PPARγ, and to see whether correlation is higher between canine and human CD for these receptors than for SS and DA. These investigations could help to fully evaluate the potential of canine CD as a direct animal model for human CD.

In conclusion, canine corticotroph adenomas obtained after transsphenoidal surgery, provide a model to study corticotroph cell (patho)physiology, due to the high yield of viable, primary tissue that retains most of its corticotroph features *in vitro*. Some distinct differences do exist, however, between human and canine corticotroph adenomas in terms of sst and D_2 receptor expression patterns and their responses to SS and DA agonists *in vitro.* These differences should be taken into account when using dogs with CD as a model to evaluate efficacy of novel somatostatin analogues and dopamine agonists for future use in human CD.

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Chapter 3

Differential regulation of human dopamine D_2 and somatostatin receptor subtype expression by glucocorticoids *in vitro*

de Bruin C, Feelders RA, Waaijers AM, van Koetsveld PM, Sprij-Mooij DM, Lamberts SW, Hofland LJ.

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ABSTRACT

Dopamine agonists and somatostatin analogues have been proposed in the treatment of ACTH-producing neuro-endocrine tumours that cause Cushing's syndrome. Inversely, glucocorticoids (GC) can differentially influence dopamine receptor D_2 or somatostatin receptor subtype (sst) expression in rodent models. If this also occurs in human neuro-endocrine cells, then cortisol-lowering therapy could directly affect the expression of these target receptors. In this study we investigated the effects of the GC dexamethasone (DEX) on D_2 and sst expression in three human neuro-endocrine cell lines: BON (carcinoid) and TT (medullary thyroid carcinoma) versus DMS (small cell lung cancer), which is severely GC-resistant. In BON and TT, sst_2 mRNA was strongly down-regulated in a dose-dependent manner (IC_{50} 0.84 nM and 0.16 nM), whereas sst_5 and especially D_2 were much more resistant to DEX-treatment. Sst_2 down-regulation was abrogated by a GC receptor antagonist and reversible in time upon GC withdrawal. At the protein level, DEX also induced a decrease in the total number of SS (-52%) and sst_2-specific (-42%) binding sites. Pre-treatment with DEX abrogated calcitonin-inhibition by sst_2-preferring analogue octreotide in TT. In DMS, DEX did not cause significant changes in expression of these receptor subtypes. In conclusion, we show that GCs selectively down-regulate sst_2, but not D_2 and only to a minor degree sst_5 in human neuro-endocrine BON and TT cells. This mechanism may also be responsible for the low expression of sst_2 in corticotroph adenomas and underwrite the current interest in sst_5 and D_2 as possible therapeutic targets for a medical treatment of Cushing's disease.

INTRODUCTION

Somatostatin (SS) and dopamine (DA) are small molecules with a variety of functions throughout the human body, including neurotransmission and inhibition of hormone release (1, 2). They bind to high-affinity receptors that belong to the family of G-protein coupled receptors. Both for SS and DA multiple receptor subtypes have been identified: sst_{1-5} and D_{1-5} (2, 3). The presence of these receptors has been demonstrated on a number of neuro-endocrine tumours and therefore, SS-analogues and DA-agonists could play an important role in the medical treatment of these tumours (4). Recent reports have shown significant efficacy of selective D_2-receptor agonists, such as cabergoline, and of a multiligand SS-analogue with high sst_5 affinity (pasireotide / SOM230) in the medical management of pituitary-dependent Cushing's syndrome (5-7). The use of sst_2-or D_2-targeting agonists has also been advocated in cases of Cushing's Syndrome due to ectopic ACTH-production by neuro-endocrine tumours (8, 9).

The hallmark of Cushing's syndrome, regardless of its cause, is a profound and sustained overproduction of glucocorticoids (GCs) by the adrenal glands. It is also known that GCs can have (in)direct effects on receptor expression patterns of different neurotransmitters and hormones in the human body. Therefore, when considering the clinical use of DA-agonists and/or SS-analogues to lower cortisol levels in a patient, it is important to investigate how alterations in glucocorticoid levels (i.e. response to treatment) can secondarily affect the expression of the target SS and DA receptors in ACTH-producing neuro-endocrine tumours. If such a (in)direct relationship exists, then this could first of all explain our current knowledge on SS and DA receptor expression patterns in human corticotroph adenomas as it has been described before by different groups (5, 10, 11). Secondly, perhaps more importantly, it could help us to predict what happens to the expression of the target SS and DA receptors, when medical therapy lowers circulating GC levels. This may have implications for the type and timing of additional SS-or DA-based medical treatment in these patients.

A considerable number of studies have investigated the effects of GCs on dopamine receptor expression, mainly within the central nervous system and associated neuro-psychiatric disorders, but not in human neuro-endocrine tumour cells (12-16). Most of these studies report no or only minor effects of GC exposure on D_2 receptor expression. Several other studies have shown that GC's can influence somatostatin receptor subtype expression in a differential manner in rat or murine pituitary cells and cell lines (17-19). No study thus far, however, has concomitantly evaluated D_2 and SS receptor subtype expression and their regulation by GC's within the same, human-derived neuro-endocrine cell system.

For that reason we conducted the present study. We investigated the time- and dose-dependent effects of the synthetic glucocorticoid dexamethasone (DEX) on sst_2, sst_5 and D_2 receptor expression *in vitro* in three different human neuro-endocrine cell lines (BON, TT and DMS-79), for which evidence of direct transcriptional regulation by GC's has been reported (20-22). BON (pancreatic carcinoid) and TT (medullary thyroid carcinoma) cells are not known to have any defects in their GC regulation and signalling pathway. By using these two different neuro-endocrine cell lines we aimed to find general patterns in GC-mediated effects that are representative for the larger group of neuro-endocrine tumours as a whole. Since BON and TT cells do not necessarily reflect ectopic ACTH-producing tumours that display severe GC-resistance, we compared these results with a third and completely different neuro-endocrine cell line: the human ectopic ACTH-producing small cell lung carcinoma cell line DMS-79, which harbours distinct mutations in the glucocorticoid receptor gene and is known for its severe GC-resistant properties (22, 23).

MATERIALS AND METHODS

Cell culture

BON cells were routinely grown in 75 cm^2 flasks containing DMEM-F12 Glutamax® medium supplemented with 10% Foetal Calf Serum, penicillin ($1x10^5$ U/l) and fungizone (0.25 mg/L). Cells were cultured in a 5% CO_2 incubator at 37°C and routinely passaged by trypsinization (trypsine 0.05% -EDTA 0.02%). Medium was refreshed twice a week and cell viability always exceeded > 95% as measured by trypan blue staining. TT cells were cultured following the same protocol with F-12K Nutrient Mixture (Kaighn's Modification). DMS cells were grown in suspension under the same conditions with 10% FCS, glutamine and penicillin in RPMI 1640 medium (ATCC 30-2001). All cell lines were confirmed to be mycoplasma-free. Media and supplements were obtained from Invitrogen (Breda, the Netherlands) unless otherwise stated.

Cell treatment for mRNA expression studies

For the experiments, cells were trypsinized, counted in a standard haemocytometer and seeded at a density of 15,000 (BON) or 100,000 (TT, DMS) cells/well in 24 well plates (Corning, Cambridge, MA, U.S.A.) in 1 ml of medium. After 72 h media were refreshed and incubations started without or with different doses of DEX and/or the GC receptor antagonist RU-486. At the different time points, media were removed and cells were lysed on ice with a buffer containing 100 mM Tris-HCl (pH 8), 500 mM LiCl, 10 mM EDTA (pH

8), 5 mM DTT and 1% LiDS (HT Biotechnology Ltd, Cambridge, U.K.) and stored at –80˚C until further analysis. All experimental conditions were performed in quadruplicates.

Quantitative PCR

Quantitative PCR was performed according to a previously published method (24). In short, poly(A^+) mRNA was isolated from the lysed cells with the use of Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway). The poly (A^+) mRNA was eluted in H_2O (65˚C) for 2 x 2 minutes and used for cDNA synthesis in a Tris buffer [50 mM Tris-HCl (pH 8.3), 100 mM KCl, 4 mM DTT and 10 mM $MgCl_2$] with 10 units RNase inhibitor, 2 units avian myeloblastosis virus Super Reverse Transcriptase and 1 mM of each deoxynucleotide triphosphate in a final volume of 40 µL. This was incubated for 1 h at 42˚C and the resulting cDNA was diluted 5-fold in 160 µl sterile H_20.

One twentieth of the total cDNA library was used for quantification of hprt, sst_2, sst_5 and D_2 mRNA levels. The total reaction volume (25 µl) consisted of 10 µl cDNA and 15 µl TaqMan Universal PCR Mastermix (Applied Biosystems, Branchburg, NJ, U.S.A.) with primers-probes in the following concentrations: hprt, sst_2 and sst_5 500-500-100 nM and D_2 300-300-200 nM of forward primer, reverse primer and probe, respectively. The primer and probe sequences that were used for hprt, sst_2, and sst_5 have been published previously (24). D_2 primer-and-probe sequences were: D_2 forward 5'-GCCACTCAGATGCTCGCC-3', D_2 reverse 5'-ATGTGTGTGATGAAGAAGGGCA-3' and D_2 probe 5'-FAM-TTGTTCTCGG CGTGT-TCATCATCTGC-TAMRA-3'. This primer-probe set measures total D_2 expression (D_2 long + short isoform). All primers and probes were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). Real-time quantitative PCR was performed in 96-well optical plates with the TaqMan Gold nuclease assay (Applied Biosystems, Roche, NJ, U.S.A.) and the ABI Prism 7700 Sequence Detection System (PerkinElmer, Foster City, CA, U.S.A.). After two initial heating steps at 50˚C (2 min) and 95˚C (10 min), samples were subjected to 40 cycles of denaturation at 95˚C (15 sec) and annealing at 60˚C (60 sec). All samples were assayed in duplicate. Values were normalized against the expression of the housekeeping gene hypoxanthine phosphoribosyltransferase (hprt). Dilution curves were constructed to calculate PCR efficiencies (E) for every primer-probe set (25). Efficiencies were: sst_2 1.92, sst_5 1.92, D_2 1.94 and hprt 1.93. Estimated copy numbers were calculated using the comparative threshold method with efficiency correction, as described previously (26). To exclude genomic DNA contamination in the RNA, the cDNA reactions were also performed without reverse transcriptase and amplified with each primer pair. To exclude contamination of the PCR reaction mixtures, the reactions were also performed in the absence of cDNA template, in parallel with cDNA samples.

Cell proliferation and DNA fragmentation assays

After trypsinization, the cells were counted, seeded and treated with DEX in similar fashion as described above in the experiments for qPCR analysis. At the different time points media were aspirated and cells were collected for DNA measurement. Measurement of total DNA contents, representative for the number of cells, was done using the bisbenzimide fluorescent dye (Hoechst 33258; Boehring Diagnostics, La Jolla, CA, U.S.A.) as previously described (27). Parallel to this, the induction of apoptosis was evaluated in these cells by DNA fragmentation analysis, using a commercially available ELISA kit (Cell Death Detection ELISA^{Plus}, Roche Diagnostics GmbH, Almere, the Netherlands) according to the manufacturer's protocol. Experiments were done in quadruplicates.

Membrane binding studies with [^{125}I-Tyr^{11}]-SS14 and [^{125}I-Tyr^{3}]-OCT

Membrane binding experiments were performed according to a previously published protocol (28). In brief, BON cells were grown to 40-50% confluency and subsequently treated with DEX 10 nM for 72 h. At 72 h cells were collected and homogenized with a Polytron Homogenizer (Kinematica). Membrane fractions were obtained by centrifugation at 14000 rpm for 30' and protein content was determined by Bradford analysis. 50µl of membrane homogenates were incubated for 45 minutes at room temperature with 25µl of increasing amounts of [^{125}I-Tyr^{11}]-SS14 (GE Healthcare, Brussels, Belgium) or [^{125}I-Tyr^{3}]-OCT (Novartis, Basel, Switzerland) tracer, with or without excess (1 µM) of unlabeled SS-14 or OCT, respectively, in 25 µl HEPES buffer (10 mM HEPES, 5 mM MgCl2, and 0.02 g/l bacitracin, pH 7.6) containing 0.2% BSA. Incubation was terminated by the addition of 1 ml of ice-cold HEPES-BSA buffer and membrane-bound radioactivity was separated from unbound by centrifugation for 2 min at 14,000 min^{-1}. The remaining pellets were washed twice in HEPES buffer, air-dried and counted in a liquid scintillation γ-counter for 1 minute. Specific binding was regarded as total binding minus the binding in the presence of excess (1µM) unlabeled SS-14 or OCT. Experimental conditions were in duplicate and experiments were performed at least twice.

Hormone release

After trypsinization, TT and DMS cells were pre-treated with DEX 10 nM or vehicle control for 72 h. Cells were then washed and subsequently treated with OCT 10 nM for 72 h. After that time, media were collected and stored at –20˚C. In DMS cells, aprotinin (4 x 10^{5} IU/ml medium; Bayer, Mijdrecht, the Netherlands) was added to the media prior to storage to prevent ACTH degradation. ACTH and calcitonin levels were measured by commercially available, non-isotopic, automatic, chemiluminescence immunoassay systems (DPC Im-

mulite, Los Angeles, CA, U.S.A.). Intra- and interassay coefficients of variation were 5.6 and 7.8% for ACTH, and 2.0 and 3.5% for calcitonin, respectively. Sensitivity thresholds were 2.0 pg/ml (calcitonin) and 5.0 pg/ml (ACTH).

Test substances

Dexamethasone and octreotide were obtained from the hospital pharmacy Erasmus Medical Center, aliquotted and stored at 4˚C. SS-14 and the glucocorticoid receptor antagonist RU-38486 (mifepristone) were obtained from Sigma Aldrich (Zwijndrecht, the Netherlands).

Statistical analysis

Each experimental condition was run in quadruplicate and experiments were performed at least twice, independently of each other. Statistical analysis was done using GraphPad Prism software Version 3.02 (San Diego, CA, U.S.A.). Average values per group were compared by ANOVA (Analysis of Variance). When significant differences were found, the Newman Keuls test was used to make comparisons between groups. IC_{50} values of dose-response curves were calculated by non-linear curve fitting. Data of membrane binding studies were analyzed by the method of Scatchard. Values of $p<0.05$ were considered statistically significant. Data are reported as mean ± SEM.

RESULTS

Baseline mRNA expression levels

The baseline expression levels of sst_2, sst_5 and D_2 mRNA in the different cell lines are depicted in figure 1. All three cell lines expressed all receptors of interest, albeit in different ratios. BON cells expressed relatively high amounts of sst_5 (0.43±0.09, mean±se), followed by D_2 (0.22±0.05) and sst_2 (0.08±0.01); TT cells predominantly expressed D_2 (1.26±0.13), followed by sst_2 (0.40±0.06) and sst_5 (0.34±0.03); DMS cells had overall lower expression levels: sst_2 (0.15±0.03), D_2 (0.12±0.01) and sst_5 (0.04±0.01).

Cell proliferation and DNA fragmentation studies

We investigated whether DEX treatment (0.1-100 nM) caused significant changes at 24 h, 72 h and 168 h in total DNA contents, hprt expression per nanogram RNA and the induction of apoptosis compared to untreated cells (data not shown). In the BON and

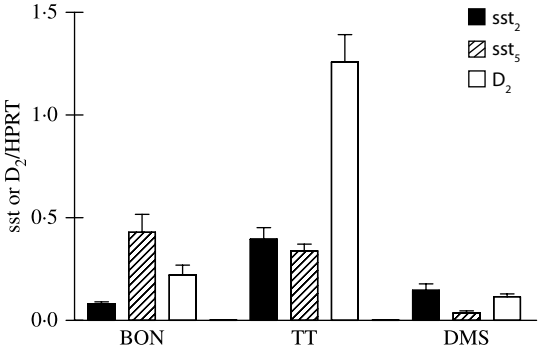

Figure 1: Sst_2, sst_5 and D_2 mRNA expression in the three different neuro-endocrine cell lines used in this study. Expression levels of receptor subtypes were normalized against the housekeeping gene hprt. Values represent the mean ± SEM of ≥ 3 independent experiments per cell line.

DMS cells we did not observe significant differences with different DEX doses at any time point. In TT cells, however, the highest DEX dose (100 nM) caused a significant decrease in DNA contents and an increase in apoptosis already after 72 h, which was not present at 24 h. For that reason we performed full dose-response experiments (0.1-100 nM) in TT cells at the 24 h time point instead of the 72 h time point, as for BON and DMS cells.

Quantitative PCR

In BON cells, sst_2 was dose-dependently down-regulated by DEX (0.1-100 nM, 72 hr) with an IC_{max} of –85% at 100 nM ($p<0.001$ vs. control) and an IC_{50} just below 1 nM (0.84 nM), see figure 2. Sst_5 was less sensitive to down-regulation with a 10-fold higher IC_{50} value (10.0 nM) than sst_2 and only showed significant down-regulation in the highest dose of DEX

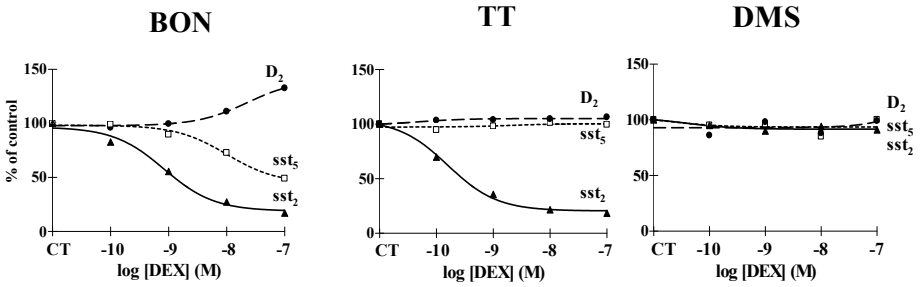

Figure 2: Dose-dependent effect of DEX treatment on sst_2, sst_5 and D_2 mRNA expression in BON, TT and DMS cells. Cells were treated with DEX (0.1-100 nM) for 72 h (BON, DMS) or 24 h (TT). Subsequently cells were lysed and sst and D_2 mRNA expression levels were determined. Expression levels were normalized against the housekeeping gene hprt. Values represent percent change relative to control. IC_{50} values were calculated for the dose-response effect of DEX on sst and D_2 mRNA expression and are indicated in the results section.

100 nM (-50%, $p<0.01$). D_2 did not show any down-regulation at any of the tested concentrations; in fact, at the highest concentration (100 nM) D_2 was slightly up-regulated (+33%, $p<0.05$). In TT cells (24 hr), a similar dose-dependent down-regulation for sst_2 was observed with an IC_{max} of –84% ($p<0.001$) and a subnanomolar IC_{50} (0.16 nM). In TT cells nor sst_5, nor D_2 showed any signs of down-regulation in any of the concentrations tested. In DMS cells (72 hr), no significant effects were observed by DEX treatment.

Based on the presence of DEX-induced effects in BON cells and the absence of growth inhibition or apoptosis induction at any treatment duration or dose, we investigated in these cells the effects of different treatment periods (24h and 168 h) on DEX-induced receptor down-regulation. We found that at 24 and 168 h, DEX-treatment produced similar dose-dependent decreases in sst_2 mRNA expression as those observed at 72h. IC_{max} and IC_{50} values were respectively: -74% ($p<0.001$) and 0.60 nM at 24 h, and -80% ($p<0.001$) and 0.84 nM at 168h, see figure 3. Also for sst_5, comparable dose-dependent effects were observed at these time points: IC_{max} -45% ($p<0.01$) and IC_{50} 4.0 nM at 24 h, and -52% ($p<0.01$) and 5.4 nM at 168 h. For D_2, no down-regulation was demonstrable at any DEX dose at these time points. The observed D_2 upregulation after 72 h with DEX 100 nM was not demonstrable at 24 h or 168h.

To investigate whether DEX-induced down-regulation of sst_2 and to a lesser extent sst_5 was a glucocorticoid receptor-specific effect, we also performed these experiments with the addition of the glucocorticoid receptor antagonist RU-38486 in all cell lines. The

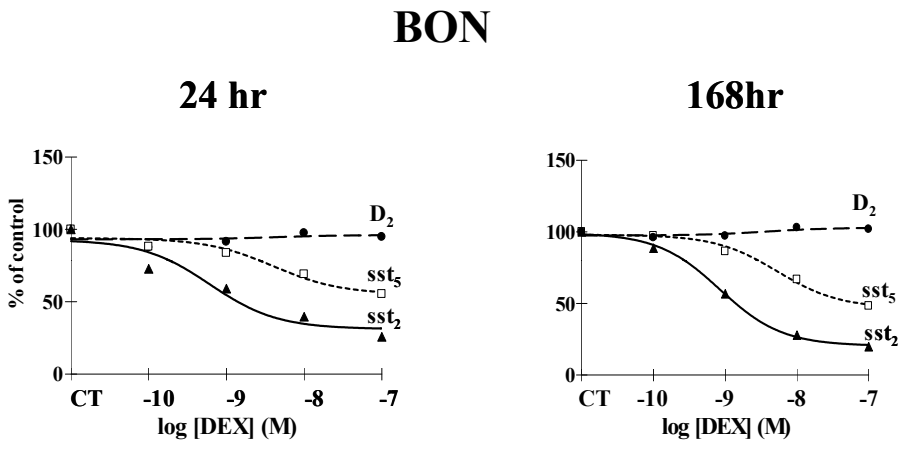

Figure 3: Effect of treatment duration (DEX) on sst_2, sst_5 and D_2 mRNA expression in BON cells. Cells were treated with DEX (0.1-100 nM) for either 24 or 168 hr. Subsequently cells were lysed and sst and D_2 mRNA expression levels were determined. Expression levels were normalized against the housekeeping gene hprt. Values represent percent change relative to control (untreated cells). IC_{50} values were calculated for the dose-response effect of DEX on sst and D_2 mRNA expression and are indicated in the results section.

down-regulation after 72 hr by DEX 10 nM of sst_2 (-71%, $p<0.001$) and sst_5 (-22%, $p<0.05$) in BON cells, of sst_2 (-69%, $p<0.001$) in TT cells, and sst_5 (-24%, $p<0.05$) in DMS cells, was completely abrogated by co-incubation with RU-38486, see figure 4.

Reversibility of GC-mediated sst_2 down-regulation was also investigated. BON and TT cells were pre-treated with DEX 10 nM for 24 h (t= Day 0). After changing the medium into DEX-free medium, the cells were subsequently cultured for another 2-4 days. After an initial sst_2 down-regulation due to DEX treatment (BON –68%, $p<0.001$; TT –66%, $p<0.001$ at Day 0), a complete reappearance of sst_2 expression was observed in both BON and TT cells after 2-4 days of culture in DEX-free medium (BON –2% after 4 days; TT +8% after 2 days, see figure 5).

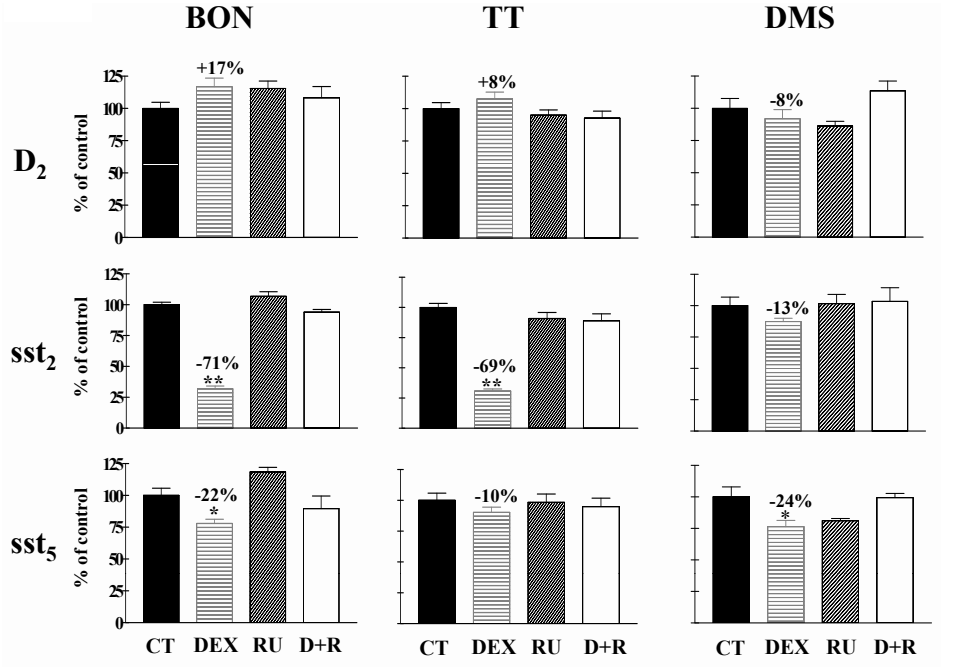

Figure 4: Antagonism of DEX-induced changes in sst_2, sst_5 and D_2 mRNA expression by co-incubation with the glucocorticoid receptor antagonist RU-38486. Cells were treated for 72 h with DEX 10 nM, RU-38486 100 nM or their combination. Subsequently, cells were lysed and sst and D_2 mRNA expression levels were determined. Expression levels were normalized against the housekeeping gene hprt. CT = control; DEX = dexamethasone 10 nM; RU = glucocorticoid antagonist RU-38486 at 100 nM (BON, TT) or 1 µM (DMS), D+R = DEX 10 nM + RU-38486; Values represent percent change ± SEM relative to control and are the mean of ≥ 2 independent experiments. *= p-value < 0.05, **= p-value < 0.001 vs. control.

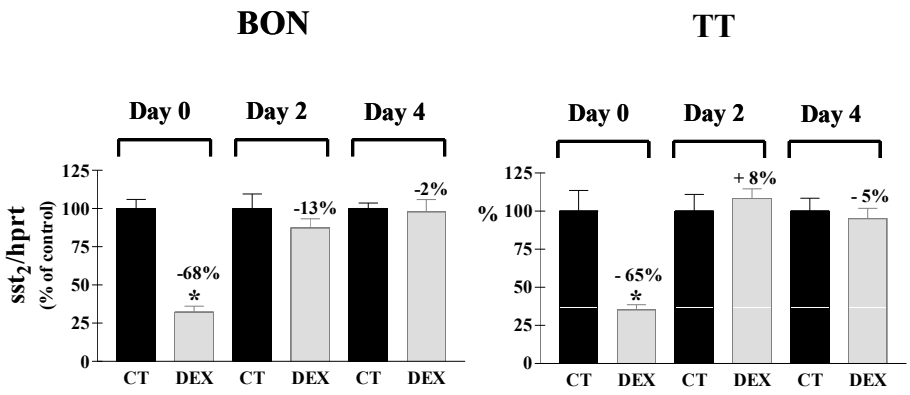

Figure 5: Reappearance of sst_2 expression after GC withdrawal in BON and TT cells. Cells were pre-treated for 24 h with DEX 10 nM. Part of the cells were lysed at that time (t= Day 0), whereas the remainder of the cells underwent prolonged culturing in normal, DEX-free medium. These cells were subsequently lysed either at Day 2 or at Day 4. Cell lysates were analyzed for sst_2 mRNA expression at the different time points. Values are represented as percent change ± SEM relative to that in the control wells on the specific time points. * = p-value <0.001 vs. control.

Membrane binding studies

To assess whether the observed sst_2 mRNA down-regulation also occurred at the protein level, membrane-binding studies were performed on BON membrane homogenates with the radiolabelled SS-analogues [^{125}I-Tyr^{11}]-SS14 and [^{125}I-Tyr^{3}]-Octreotide. Treatment of BON cells with DEX 10 nM for 72 h led to a 42% decrease in total sst receptor binding sites, as measured by [^{125}I-Tyr^{11}]-SS14 binding: B_{max} (DEX) 60 fmol/mg vs. B_{max} (control) 104 fmol/mg with unchanged binding affinity (K_d 1.2 nM), see figure 6a. The decrease in total sst binding sites included a marked decrease (-52%) in specific sst_2-binding sites as shown by [^{125}I-Tyr^{3}]-Octreotide binding: B_{max} (DEX): 40 fmol/mg vs. B_{max} (Control): 84 fmol/mg, with unchanged binding affinity (K_d 0.2 nM, see figure 6b).

Hormone release data

In TT cells, 72 h treatment with the sst_2-preferring analogue octreotide 10 nM induced a significant decrease in calcitonin release compared to control (-32%, $p<0.001$, see figure 7). However, when TT cells were pre-treated with DEX 10 nM for 72 h, the octreotide-mediated inhibition of calcitonin release was completely abolished (-3%, $p>0.05$). In DMS cells, treatment with OCT 10 nM for 72 h did not significantly inhibit ACTH release in either control or DEX pre-treated cells.

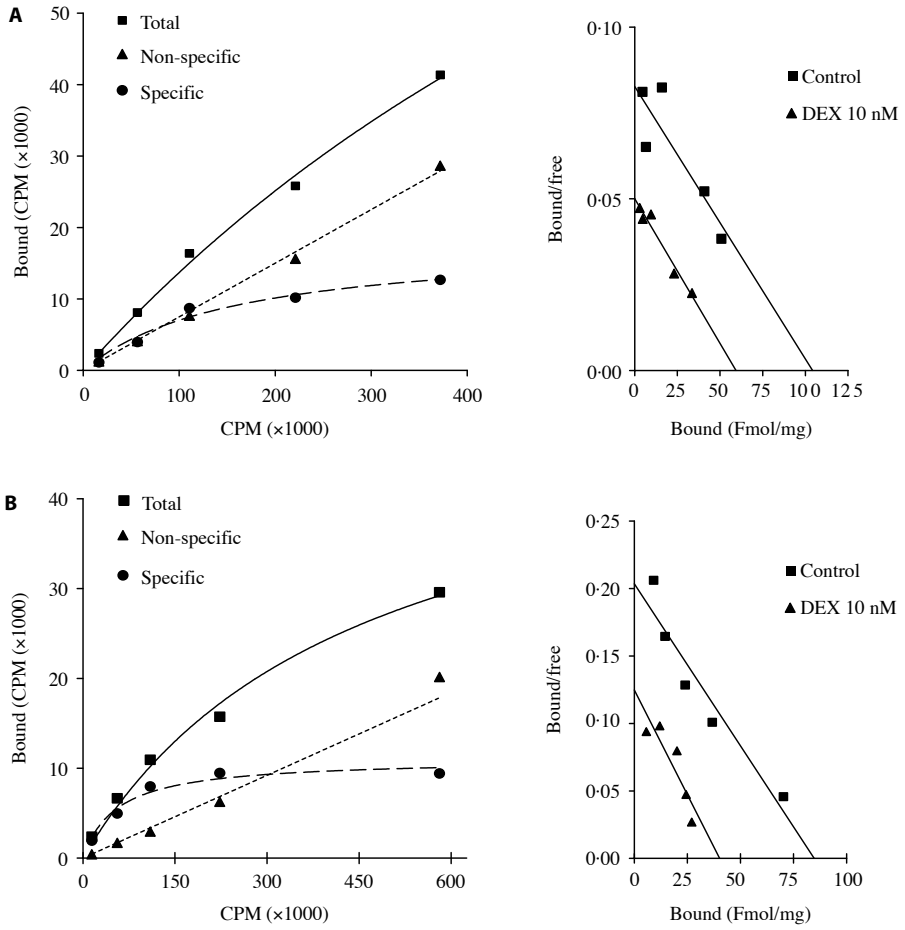

Figure 6: Membrane-binding studies. *A*: Scatchard analysis of [^{125}I-Tyr^{11}]-SS-14 binding to cell membranes of BON cells, cultured in the absence (*K*d 1.2 nM, B_{max} 104 fmol/mg) or presence of 10 nM DEX (72 h; *K*d 1.2 nM, B_{max} 60 fmol/mg). *B*: Scatchard analysis of [^{125}I-Tyr^{3}]-OCT binding to cell membranes of BON cells cultured in the absence (*K*d 0.2 nM, B_{max} 84 fmol/mg) or presence of 10 nM DEX (72 h; *K*d 0.2 nM, B_{max} 40 fmol/mg). ■, Control; ▲ 10 nM DEX.

DISCUSSION

In this study we have shown that somatostatin receptor subtypes (sst) and the dopamine D_2 receptor, natively co-expressed by human neuro-endocrine cell lines, show a differential pattern of response to glucocorticoid exposure *in vitro*.

In both BON and TT cells, sst_2 is highly sensitive to GC-induced down-regulation in a dose-dependent manner, whereas the sst_5 is significantly less sensitive. Moreover, D_2 is fully insensitive to this type of down-regulation. The phenomenon appears to be

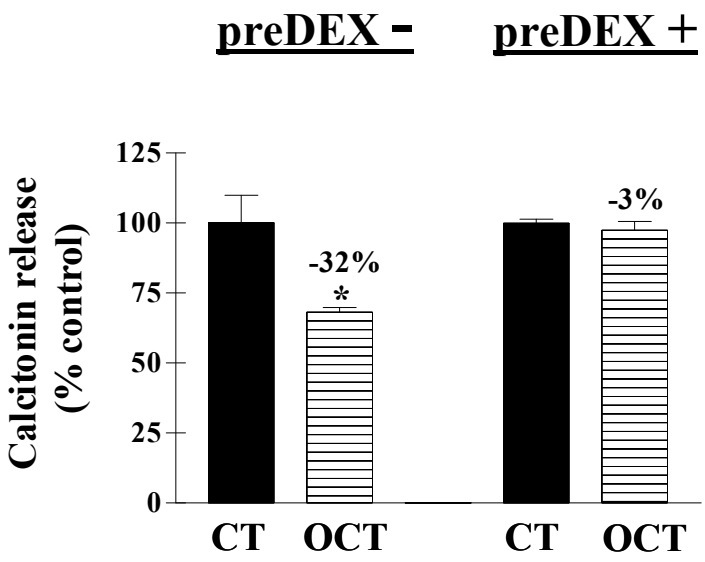

Figure 7: Effect of DEX pre-treatment on inhibition of hormone release by octreotide in TT cells. Cells were pre-treated for 72 h with DEX 10 nM (preDEX+) or not (preDEX-). At that time (t=0), cells were washed, media refreshed and incubations started for 72 h with octreotide 10 nM. After 72 h, media were aspirated and analyzed for calcitonin levels. All experiments were performed in quadruplicates. Values represent percent change relative to control (= no OCT treatment). * = p-value < 0.001 vs. control. CT= control; OCT = octreotide.

glucocorticoid receptor (GR)-specific, as addition of the GR antagonist RU-38486 can completely abrogate these effects and because sst_2 down-regulation is fully reversible in time upon GC withdrawal. The functional relevance of this sst_2 down-regulation could be demonstrated by the fact that the sst_2-preferring analogue octreotide lost its efficacy in DEX pre-treated TT cells. In DMS cells, derived from a severely GC-resistant tumour type in vivo, these GC-mediated effects on sst_2 expression were not demonstrable.

The results in BON and TT cells could indirectly explain the low sst_2 expression found specifically in human corticotroph adenomas (10, 11), whereas this receptor subtype is abundantly expressed in other pituitary adenomas, such as somatotroph and non-functioning adenomas (29, 30). Based on our results, one can speculate that high endogenous GC levels in patients with Cushing's disease (CD) are at least partially responsible for this observed difference in sst_2 expression between adenoma types. It would also explain the low clinical efficacy of octreotide in the treatment of CD: most studies on the use of this sst_2-preferring compound in CD report no to limited effect (31-33).

At the same time, these data do support the interest in the potential use of sst_5 and D_2 selective agents in the medical treatment of CD. Apparently, these receptor subtypes are fully (D_2) or partially (sst_5) resistant to high GC pressure and thus may still be of

functional value in the regulation of ACTH release in corticotroph adenomatous cells. Several clinical studies have been performed already with these agents and have shown promising results in subsets of patients. Pivonello et al. showed that 3-month treatment with the D_2-specific agonist cabergoline could significantly reduce urinary free cortisol (UFC) levels in 60% of patients and even induce complete normalization of UFC in 40% of patients with CD (5). An early report on the use of the multi-ligand SS analogue pasireotide (SOM230) which has subnanomolar affinity for sst_2, sst_3 and sst_5, showed equally interesting results: 15-day treatment with pasireotide 600µg twice daily, led to a complete normalization of UFC in 19% (4/21) and a ≥ 50% decrease in UFC in another 24% (5/21) of CD patients, probably mediated through sst_5 receptor activation (6). Considering the reported efficacy of both cabergoline and pasireotide in subsets of CD patients, combination treatment with these agents is an interesting future option. For that reason, studying the effects of GC on a single cell system from a neuro-endocrine origin that expresses sst_2, sst_5 and D_2, could help us understand some more of the biological backgrounds of these clinical observations.

This is the first study to describe a differential pattern of GC-responsiveness regarding both D_2 and SS receptor subtypes co-expressed in human neuro-endocrine cell lines. The observed pattern closely resembles the one seen earlier in the murine corticotroph AtT-20 cells (19). In these cells, sst_2 is sensitive whereas sst_5 is largely insensitive to GC-induced down-regulation. Unfortunately, D_2 was not expressed at sufficiently high levels in these cells to allow for investigation of this receptor subtype (unpublished observations). Nevertheless, one could hypothesize that a similar pattern of GC-responsiveness exists in different human and murine neuro-endocrine cell lines.

Our findings are in line with earlier studies that describe the presence of distinct GC-responsive elements (GREs) in the murine sst_2 gene, whereas the murine sst_5 gene only contains multiple GRE-half sites (34-36). Moreover, in transfection experiments it was shown that the human sst_2 gene promoter is under direct control of GC's, whereas the sst_5 promotor is not (37, 38). To our knowledge, comparable GC-responsive regulatory sequences have thus far not been identified within the D_2 gene.

Previously, Petersenn et al. found in GH4 cells (rat pituitary adenoma cell line) that hydrocortisone 100 nM did not influence sst_5 promoter activity whereas we do find moderate but significant down-regulation of sst_5 at higher DEX doses (100 nM) in BON cells (37). We believe that this difference can be ascribed to the difference in relative *in vitro* potency of hydrocortisone compared to dexamethasone. In previous studies on differential regulatory mechanisms by glucocorticoids it was found that DEX has a 14-fold higher potency *in vitro* compared to hydrocortisone (GILZ EC_{50} of 4.1 and 56.7 nM,

respectively) (39). Therefore, the dose used by Petersenn et al. would be the equivalent of approximately 7 nM DEX in our study. At this dose we did not observe any significant effects on sst_5 mRNA expression either.

Even though corticotroph adenoma cells are GC-resistant to a certain degree, GC regulation of SS receptors apparently remains intact in AtT-20 cells. Interestingly, BON and TT cells, derived from tumours which do not show GC-resistance in vivo, have indeed a higher degree of sst_2 down-regulation when exposed to DEX 10 nM, compared with the partially GC-resistant corticotroph adenoma cell line AtT-20 (19). In the severely GC-resistant DMS cells, derived from an ectopic ACTH-producing small cell lung cancer, most of the GC-induced sst_2 down-regulation is lost, as would be expected. The latter *in vitro* observation correlates with two interesting clinical observations. First of all, many ectopic ACTH-producing tumours are positive on ^{111}In-pentreotide receptor scintigraphy (OctreoScan) (40-43), despite the hypercortisolistic environment they are exposed to. And secondly, octreotide has been shown to be effective in controlling tumour size and cortisol production in some of these patients as opposed to patients with pituitary-derived CD (44, 45).

Another aspect we have observed in this study is the reappearance of sst_2 expression within 2-4 days after withdrawing GC-exposure. This observation could be of clinical interest. When it is possible to lower GC levels in CD patients with the use of for instance sst_5 or D_2 selective agents, then this state of normocortisolemia could lead to a re-expression of sst_2 in the corticotroph adenomas of these patients. Return of sst_2 expression in vivo would result in a strong increase in efficacy of traditional SS analogs such as octreotide (sst_2) as well as pasireotide (sst_2+sst_5) and thus expand the pharmacological options to maximally inhibit ACTH production. Obviously, the question remains to which extent these *in vitro* observations can model the clinical situation of a CD patient in whom long-term GC-overexposure is relieved through cortisol-lowering therapy. Clinical evidence in favour of a return of sst_2 expression in vivo upon GC withdrawal does exist, however. In patients with Nelson's Syndrome, inoperable or recurrent corticotroph adenomas (low sst_2 expression) necessitate bilateral adrenalectomy, leading to chronic hypo-/ or normocortisolemia. By removing most of the excessive negative feedback loop on the pituitary, the ACTH-producing pituitary adenomas left *in situ* may expand with time and can even show invasive growth in surrounding tissues. Some of these Nelson adenomas, however, are visible on ^{111}In-pentreotide receptor scintigraphy (OctreoScan), whereas most primary corticotroph adenomas are not (46, 47). Moreover, octreotide has been effective in some of these Nelson patients by lowering ACTH levels and stabilizing tumour growth (31, 48, 49). Most likely, this reappearance of functional sst_2 receptor expression is a direct effect of removing chronic hypercortisolism in these patients.

In conclusion, we show that GCs selectively down-regulate sst_2, but not D_2 and only to a minor degree sst_5 in human neuro-endocrine BON and TT cells. If this is a common regulatory mechanism in human neuro-endocrine cells, then these data would support the hypothesis that chronically elevated GC levels in Cushing's disease may be directly responsible for the low expression of sst_2 in corticotroph adenomas. It also suggests that sst_5 and D_2 are interesting candidate receptors in the search for a medical treatment of CD, due to their (relative) resistance to GC-induced down-regulation. It needs to be emphasized, however, that these data require confirmation, preferably in primary cultures of human corticotroph adenomas.

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Chapter 4

Co-expression of dopamine and somatostatin receptor subtypes in corticotroph adenomas

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ABSTRACT:

Context: Previous studies have demonstrated the expression of somatostatin receptor subtypes (mainly sst_5) and dopamine (DA) receptor subtypes (mainly D_2) in smaller series of human corticotroph adenomas. In line with these findings, sst_5 and D_2-targeting agents have already been used clinically in patients with Cushing's Disease (CD) and have shown promising results in subsets of patients. To what extent these receptor subtypes are co-expressed within individual adenomas, is not known however.

Objective: To investigate the (co-)expression of both sst and DA receptors in a large series of human corticotroph adenomas.

Design: In vitro analysis of corticotroph adenoma tissue obtained via transsphenoidal adenomectomy

Setting: Two university medical centers

Patients: 30 patients with Cushing's disease

Results: Analyzed by quantitative RT-PCR, D_2 and sst_5 were significantly (co-)expressed in the majority (60%) of adenomas, whereas 23% of adenomas only expressed D_2, but not sst_5. The remaining 17% of adenomas did not significantly express either sst_5 or D_2. Overall, expression of sst_{1-4} and D_4 was low to non-detectable. Corticotroph adenomas with invasive growth invariably showed loss of sst_5 and D_2 expression. Autoradiography revealed clear D_2 and/or SS-14 binding in a subset of cases, which correlated well with their respective mRNA data.

Conclusions: Sst_5 and especially D_2 are highly expressed in the majority of human corticotroph adenomas with co-expression of sst_5 and D_2 being a common phenomenon. These findings support the current studies with sst_5 and D_2-targeting agents in patients with CD and highlight the rationale behind sst_5-D_2 combination therapy.

INTRODUCTION:

Cushing's disease (CD) is a severe endocrine disorder, caused by an ACTH-producing pituitary adenoma. Patients suffer from the effects of chronic hypercortisolism, which can lead to hypertension, diabetes mellitus, osteoporosis and psychiatric disturbances among others (1). First line of treatment is transsphenoidal selective adenomectomy, but even in experienced hands long-term cure does not exceed 70% (2). In cases of persistent or recurrent CD, re-operation, radiotherapy and/or bilateral adrenalectomy can be performed, but these treatment options all have significant disadvantages, including the risk of permanent hypopituitarism and the lifelong dependence on hormonal replacement therapy. Until now, medical therapies with cortisol-lowering drugs such as ketoconazole or metyrapone, have not been shown to be both effective and safe for the long-term treatment of CD (3).

In recent years, however, some interesting new advances have been made in the field of receptor physiology in CD. It was found that the dopamine receptor subtype 2 (D_2) is expressed in approximately 80% of human corticotroph adenomas and that these adenomas can be responsive to the ACTH-inhibiting actions of D_2-agonists *in vitro* (4). Moreover, treatment for three months with the D_2-selective agonist cabergoline induced complete normalization of 24 hr urinary free cortisol (UFC) in approximately 40% of CD patients (4). This percentage of complete responders appears to be maintained after two years of therapy (5). At the same time, different groups demonstrated that most human corticotroph adenomas strongly express somatostatin (SS) receptor subtype 5 (sst_5) and that SS-analogues with high-binding affinity for sst_5, such as the SS-multiligand pasireotide (SOM230), can inhibit ACTH-release and cellular proliferation *in vitro* (6, 7). The cortisol-lowering effects of pasireotide *in vivo* have recently been demonstrated in a phase II clinical trial in patients with CD (8).

Based on these findings, medical therapy with D_2-agonists and/or SS-analogues with sst_5-affinity appears to be an interesting treatment option in cases of persistent or recurrent CD. To our knowledge, no study has investigated the expression of both SS and DA receptors within the same set of human corticotroph adenomas. A better understanding of the (co-)expression patterns of these two receptor subtypes could be of clinical importance. First of all this could help predict the overall percentage of corticotroph adenomas that possess a molecular target for SS and/or DA directed medical therapy. At present it is not known whether the D_2–positive and sst_5-positive adenomas in the previously mentioned studies largely overlap or whether they constitute separate groups that require different therapies. Secondly, since Rocheville et al. (9) published their report on heterodimerization of SS and DA receptors and resulting synergism in

effect, combined treatment with DA agonists and SS analogues has been an important research topic in pituitary adenomas. It has also led to the pharmaceutical development of "dopastatin" chimeras: chemically engineered compounds that possess high binding affinity for both SS and DA receptor subtypes and which may display superagonistic properties compared with traditional SS analogues and DA agonists (10).

For that reason, our main study aim was to characterize the co-expression of dopamine and somatostatin receptor subtypes in a large series of human corticotroph adenomas to gain a better understanding of the distribution of the primary molecular targets for DA/SS based medical therapy in CD. In addition, we aimed to correlate the DA/SS receptor expression patterns in these adenomas with the main biochemical and clinical parameters of HPA-axis over-activation in the corresponding CD patients.

MATERIALS AND METHODS

Corticotroph adenoma tissue

Human corticotroph adenoma tissue was available from patients with CD who underwent transsphenoidal selective adenomectomy in the period between 1991 and 2007 in one of the two participating academic centers. Informed consent was obtained from all patients of whom we assessed receptor expression for investigational purposes. The diagnosis of CD was based on clinical signs, elevated 24 hr UFC excretion, incomplete suppression of serum cortisol by 1-mg dexamethasone and/or the absence of a diurnal cortisol rhythm. In all patients imaging (MRI with iv contrast) or inferior sinus petrosus sampling confirmed the pituitary source of ACTH overproduction. Normal anterior pituitary tissue was obtained at autopsy from two patients who had died from non-endocrine diseases.

Purity of corticotroph tissue

At the study start, the corticotroph adenoma specimens were transferred into Tissue Tek Compound (Sakura Finetek, Torrance, CA) at –80˚C for further handling. From these tissues, cryostat sections were cut at 5 µm. Sections from the center of the adenoma were used for an initial immunohistochemistry (IHC) step to check for ACTH expression (see below for protocol) and a sequential haematoxylin-and-eosin stain to demonstrate the histological presence of adenoma tissue. In samples where both of these criteria were sufficiently met, directly adjacent tissue sections were cut at 20µm, from which RNA was extracted for further quantitative PCR analysis of dopamine and somatostatin

receptor subtype expression (see below for qPCR methods). On average, 5-10 sequential sections (of 20µm each) per adenoma were needed to obtain sufficient amounts of RNA. Through this approach of sequential sections we aimed to obtain pure corticotroph adenoma RNA and limit the potential interference by normal anterior pituitary tissue surrounding the adenoma. As a secondary check for purity of corticotroph tissue, we assessed GH/POMC mRNA expression ratios in all corticotroph adenoma samples and compared these with the GH/POMC ratio in the normal human anterior pituitaries. GH/POMC ratios of less than 10% compared with normal pituitaries were regarded as pure corticotroph adenoma tissues; GH/POMC ratios of 10% or more were regarded as significantly contaminated with normal pituitary tissue and were excluded from further analysis in the pure corticotroph adenoma group (11).

Quantitative PCR

Expression analysis was performed by quantitative PCR, according to a previously described method (11, 12). The sequences and final concentrations of the hprt and sst_{1-5} primer-probe pairs have been described previously (6, 12). The sequence of the sst_4 probe contains a single nucleotide polymorphism at one position. The other primer and probe sequences are depicted in Table 1. Samples were measured on an ABI Prism 7900 Sequence Detection System (Perkin Elmer, Foster City, CA) and normalized against the expression of the housekeeping gene hprt. PCR efficiencies (E) were calculated for every primer-probe set and varied between 1.91 and 2.00 (13). Estimated copy numbers

Table 1 Primer-probe sequences for D_2, D_{2L}, D_4, GH and POMC

Primer	Sequence 5'-3'	Efficiency
D_2 forw	GCCACTCAGATGCTCGCC	1.94
D_2 rev	ATGTGTGTGATGAAGAAGGGCA	
D_2 pr	FAM-TTGTTCTCGGCGTGTTCATCATCTGC-TAMRA	
D_{2L} forw	CCCACCTGAGGGCTCCA	1.95
D_{2L} rev	TGATAACGGTGCAGAGTTTCATG	
D_{2L} pr	FAM-TAAAGGGCAACTGTACTCACCCCGAGG-TAMRA	
D_4 forw	TGCACCGCCTCCATCTTC	2.00
D_4 rev	CCACGAACCTGTCCACGC	
D_4 pr	FAM-ACCTGTGCGCCATC-TAMRA	
GH forw	GCCCATCGTCTGCACCAG	1.96
GH rev	GGGATATAGGCTTCTTCAAACTCCT	
GH pr	FAM-TGGCCTTTGACACCTAC-TAMRA	
POMC forw	GAGCAGCCAGTGTCAGGACC	1.92
POMC rev	CGGATGCACTCCAGCAGG	
POMC pr	FAM-CACCACGGAAAGCA-TAMRA	

were calculated using the comparative threshold method with efficiency correction, as described previously (14). Expression of the D_2 short isoform was calculated as D_2 total minus D_2 long isoform.

Immunohistochemistry

The expression of ACTH in the corticotroph adenoma tissues was assessed by cutting cryostat sections (5µm) and performing immunohistochemistry according to a previously published method (15). The anti-ACTH primary antibody (Santa Cruz, mouse monoclonal) was used at a dilution of 1:100.

Autoradiography

In a number of cases (n=9), sufficient adenoma tissue was available to perform additional receptor autoradiography, according to previously published protocols (4, 15). Slides were incubated with either the D_2 receptor antagonist ^{125}I-sulpiride or the SS (sst_{1-5}) analogue ^{11}Tyr-SS-14 (both from GE Healthcare, Brussels, Belgium) in the absence or presence of unlabeled cabergoline 10^{-6} M (Pharmacia, Milan, Italy) or somatostatin-14 10^{-6} M (Sigma, Zwijndrecht, the Netherlands), respectively. The sections were exposed to phosphor imaging screens for 16 days in x-ray cassettes and analyzed using a Cyclone phosphor imager (Packard Instruments Co, Groningen, The Netherlands). The level of receptor binding was scored as: absent (-); weak or focal positivity (+); strong positivity (++).

Clinical data

Patient charts were retrieved to collect data on various clinical, biochemical and radiological parameters, see Table 2. Adenoma size and growth characteristics were categorized according to the modified Wilson classification system (16). Baseline mean 24 hr UFC values were documented at the time of initial diagnosis, before treatment with ketoconazole or metyrapone was initiated. For the determination of serum cortisol, plasma ACTH, serum prolactin and urinary free cortisol, different assays have been used in the two participating centers over the course of the study period (1991-2007). We expressed these parameters as a ratio of the upper limit of normal (ULN) of the specific assay.

Table 2 Patient characteristics (n=30)

Parameters	Value (% or mean±sd)	Range
Clinical characteristics		
Female sex (%)	83	
Age (yrs)	42.1	14-68
BMI (kg/m^2)	28.9	20.5-48.4
SBP (mm Hg)	154	120-240
DBP (mm Hg)	93	75-115
Pre-treatment received[1] (%)	86	
Duration pre-treatment (months)	3	1-7
Macroadenoma ≥10 mm (%)	50	
Adenoma grade (modified Wilson):[2]		
- Grade 1	43	
- Grade 2	33	
- Grade 3	7	
- Grade 4	17	
Pre-operative[3]		
UFC 24 hr (xULN):	4.6 ± 4.1	0.60-18.7
Cortisol serum (xULN):		
- mean diurnal[4]	1.04 ± 0.44	0.50-2.07
- evening/morning	0.97 ± 0.34	0.57-2.10
- 8 a.m.	1.15 ± 0.46	0.43-1.90
- after DEX 1 mg[5]	0.99 ± 0.74	0.09-2.63
- after CRH 100 µg.[5]	1.65 ± 0.77	0.68-3.80
- after DEX 7 mg[5]	0.51 ± 0.48	0.05-1.75
ACTH plasma (xULN):		
- mean diurnal[4]	1.87 ± 2.06	0.33-9.13
- 8 a.m.	1.31 ± 1.00	0.40-3.41
- after CRH 100 µg[5]	2.11 ± 1.51	0.76-6.07
IGF-1 (nmol/l)	23.9 ± 10.5	5-46
PRL (xULN)	0.90 ± 1.27	0.18-5.77
Fasting blood glucose (mmol/l)	5.8 ± 2.3	3.7-14.4
Post-operative		
% Remission	72	
Cortisol serum 8 a.m. (xULN)	0.46 ± 0.39	0.02-1.32
Fasting blood glucose (mmol/l)	5.3 ± 1.2	3.6-8.0
Serum PRL (xULN)	0.55 ± 1.11	0.00-5.97
Follow-up (months)	55.2	0-150
Hydrocortisone dependence (months)	14.6	0-62

[1] Pre-operative treatment consisted of either ketoconazole or metyrapone monotherapy.
[2] Modified Wilson classification: Grade 1 (noninvasive microadenoma), Grade 2 (noninvasive macroadenoma), Grade 3 (invasive microadenoma), Grade 4 (invasive macroadenoma)
[3] Laboratory parameters are expressed relative to the upper limit of normal (ULN) of the individual assay (1.0 = value at the level of ULN), except for fasting blood glucose (ref 3.5-5.6 mmol/l) and IGF-1 (reference values age- and gender-specific).
[4] Mean diurnal serum cortisol and ACTH were calculated as the average of the 8 a.m, 5 p.m. and 10 p.m. values. [5] The resulting maximal (after CRH 100 µg iv) and minimal serum cortisol levels (after DEX 1 mg p.o. or 7 mg iv) are reported relative to the standard ULN of serum cortisol.

Statistical analysis

SPSS for Windows 16.0 (SPSS Inc., Chicago, IL) was used for statistical analyses. Receptor expression levels are expressed as mean ± sd (or as median + range). Univariate analysis was performed between individual receptor expression levels and clinical parameters to calculate Spearman's correlation coefficients, since the data were not normally distributed. For categorical variables the Chi-Square test was performed.

RESULTS

Purity of corticotroph tissue and patient inclusion

Frozen corticotroph adenoma tissue was available from 48 different CD patients. Six of these 48 tissues did not display adequate ACTH+ staining on IHC or had an insufficient yield of mRNA and were therefore discarded. Twelve of the remaining 42 tissues had a GH/POMC mRNA expression ratio of >10% of the level found in normal human anterior pituitary (mean ± sd of GH/POMC mRNA copy number ratio in normal anterior pituitary: 410 ± 17). We regarded these 12 tissues as corticotroph adenomas with a significant degree of contamination with normal surrounding anterior pituitary tissue and these were also excluded. Therefore, a final group of 30 pure corticotroph adenomas remained for further analysis in this study.

The clinical characteristics of the 30 CD patients from whom we received pure corticotroph adenoma tissue, are depicted in Table 2. Mean age was 42 yrs (range 14-68) and 83% were female. The majority of patients (86%) had received pre-operative treatment with cortisol-lowering drugs (ketoconazole or metyrapone) for an average duration of 3 months. In our study population, 43% of cases were non-invasive microadenomas (Grade 1), whereas the remainder of the cases (57%) were adenomas with a Wilson classification Grade 2 or higher. Overall remission rate was 72% in this series with a mean follow-up of 55.2 months.

Mean sst and DA expression levels

The mean mRNA expression levels of sst and DA receptor subtypes in the corticotroph adenomas are shown in Figure 1A and 1B. Among the different SS receptor subtypes, sst_5 was most predominantly expressed with a mean of 0.136 ± 0.028 copy numbers relative to hprt (median 0.107), while sst_2 was expressed at lower levels with a mean of 0.043 ± 0.012 (median: 0.020). Sst_1 (mean 0.024 ± 0.009; median 0.004), sst_3 (mean 0.004

± 0.002; median 0.000) and sst_4 expression (mean: 0.021 ± 0.007; median: 0.009) was very low (Fig 1A).

With respect to DA (D_2-like) receptors, total D_2 expression was high with a mean of 1.30 ± 0.37 copy numbers relative to hprt (median: 0.22). The majority (78%) of D_2 expression was of the D_2 long subtype (mean 1.01 ± 0.27). The D_4 subtype was not detectable in this series of corticotroph adenomas (Fig 1B).

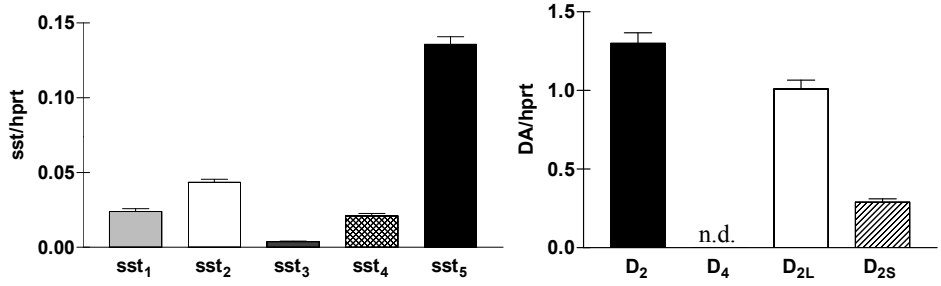

Figure 1: Mean expression levels of somatostatin (fig 1A) and dopamine (Fig 1B) receptor subtypes in 30 human corticotroph adenomas. Values represent the mean ± SEM per receptor subtype, assayed in duplicate. Expression levels are normalized against the housekeeping gene hprt. D_{2L} and D_{2S} are the long and short isoforms of the D_2 receptor, respectively. N.d. = not detectable

Co-expression of sst_2, sst_5 and D_2

The mRNA expression data of sst_2, sst_5 and D_2 in the individual corticotroph adenomas C1-C30 is depicted in Figure 2. In 60% of cases (18/30) co-expression of sst_5 and D_2 mRNA was observed, with the use of 0.04 (sst/hprt) and 0.07 (D_2/hprt) as a threshold value for significance (see below at autoradiography for the calculation of these threshold values). In 23% of cases (7/30) significant D_2 expression was found, but not of sst_5. In the remaining 17% of cases (5/30), no significant expression of either sst_5 or D_2 was found. Sst_2 expression was generally much lower than that of sst_5 or D_2, but was present at significant levels in 30% (9/30) of adenomas.

Adenoma grade and sst/D_2 expression

In Figure 3 the mean sst and D_2 mRNA expression levels are depicted, classified by adenoma grade (Wilson classification). This figure shows that in grade 1 noninvasive microadenomas (n=13), mean (±SEM) expression levels were relatively high: 1.83 ± 0.55 (D_2), 0.22 ± 0.05 (sst_5) and 0.07 ± 0.02 (sst_2). In grade 2 adenomas (n=10), which are noninvasive macroadenomas, similar expression levels were found, albeit somewhat lower: 1.49 ± 0.80 (D_2), 0.12 ± 0.04 (sst_5) and 0.02 ± 0.01 (sst_2). However, in grade 3 (n=2) and grade 4 (n=5) adenomas, which exhibit invasive growth into surrounding structures,

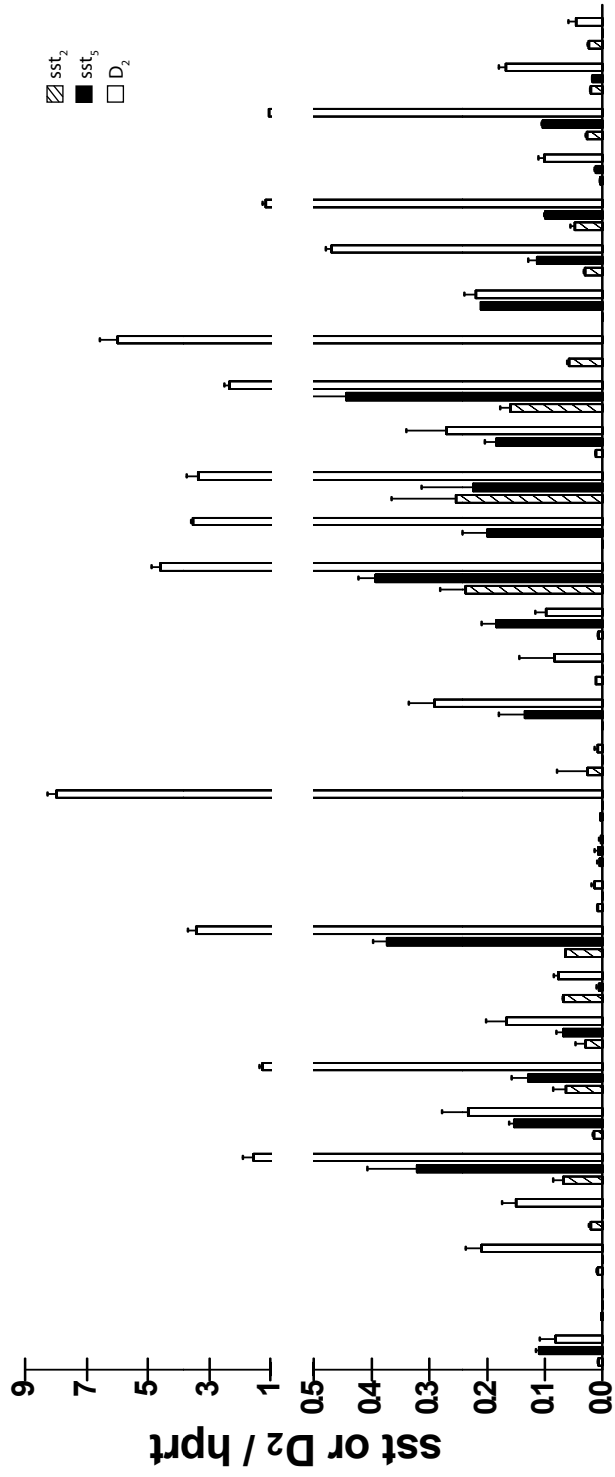

Figure 2: Overview of sst_2, sst_5 and D_2 mRNA expression in adenoma cases C1-C30. All expression levels are normalized against the housekeeping gene hprt. Values represent the mean ± SEM of two duplicate measurements per adenoma.

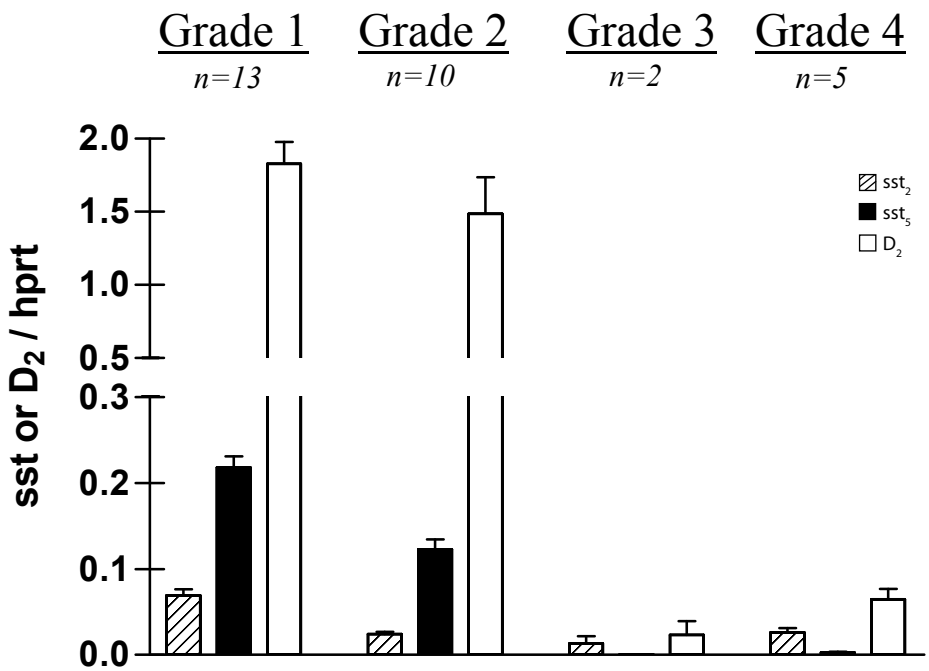

Figure 3: Mean sst_2, sst_5 and D_2 mRNA expression per adenoma grade. The modified Wilson classification was used. Grade 1: noninvasive microadenoma; Grade 2: noninvasive macroadenoma; Grade 3: invasive microadenoma; Grade 4: invasive macroadenoma. Expression levels are normalized against the housekeeping gene hprt. Values represent the mean expression levels ± SEM per receptor subtype per adenoma grade.

sst_5 and D_2 receptor expression was extremely low: 0.02 ± 0.02 (D_2), 0.00 ± 0.00 (sst_5) and 0.01 ± 0.01 (sst_2) for grade 3, and 0.06 ± 0.03 (D_2), 0.00 ± 0.00 (sst_5) and 0.03 ± 0.01 (sst_2) for grade 4 adenomas.

Autoradiography

In 9 cases we had sufficient tissue to perform additional autoradiography. In the majority of these tissues (7/9), there was a good correlation between the levels of sst and D_2 mRNA expression and the degree of ^{111}Tyr-SS-14 (sst_{1-5} agonist) and ^{125}I-sulpiride (D_2-antagonist) binding at autoradiography, see Figure 4A. Receptor binding levels were scored as absent (-), weakly/focally positive (+) or strongly positive (++). The average mRNA expression levels per category of receptor binding were: 0.01 (-), 0.04 (+) and 0.13 (++) for sst/hprt and 0.01 (-), 0.07 (+) and 3.07 (++) for D_2/hprt. The resulting cut-off point between no binding (-) and demonstrable protein binding (+ or ++) was used to assess significance of mRNA expression (≥0.04 for sst/hprt, ≥0.07 for D_2/hprt), as described earlier for the mRNA co-expression data. Two exemplary cases (C13 and C28) of autoradiography are shown in Figure 4B. C13 only had demonstrable D_2 expression

Fig. 4A

Case	^{11}Tyr-SS-14	^{125}I-sulpiride
C1	++	+
C2	-	-
C9	+	+
C11	-	-
C13	-	++
C16	+	+
C28	++	++
C29	-	++
C30	+	+

Fig. 4B

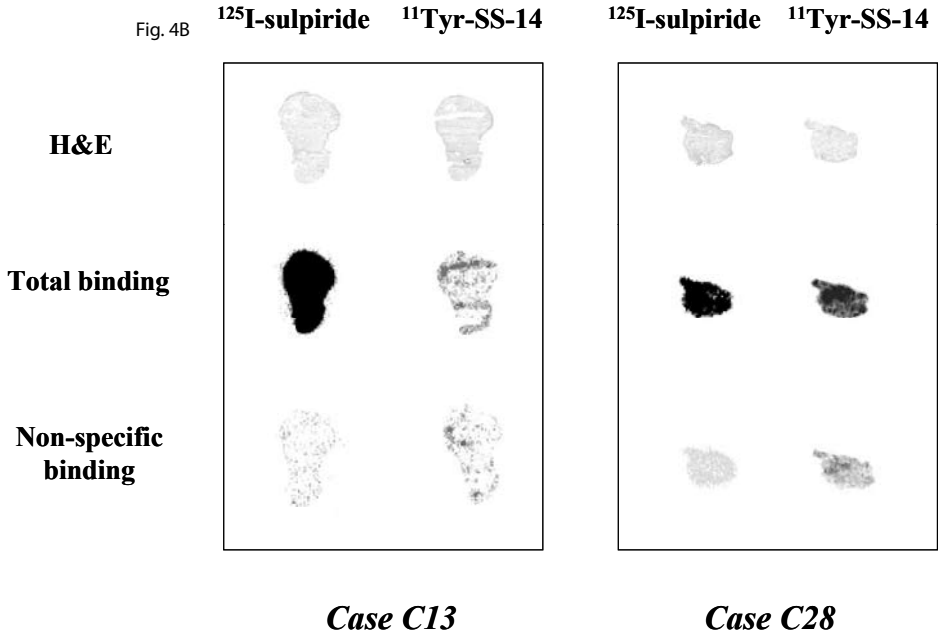

Figure 4: Autoradiography on corticotroph adenomas for D_2 (^{125}I-sulpiride) and SS-14 (^{11}Tyr-SS-14) receptor binding. Fig 4A: Overview of autoradiography results in the 9 available cases. Receptor binding was classified as: absent (-); weak or focal positivity (+); strong positivity (++). Fig 4B: Two exemplary cases (C13 and C28): Case C13 shows high specific (i.e. total – aspecific) binding for D_2, but not for SS-14. Case C28 shows specific binding for both D_2 and SS-14.

at the mRNA level, which correlated with the observation that only ^{125}I-sulpiride showed specific binding at autoradiography. Case C28 expressed significant levels of both sst_5

and D_2 mRNA, which was in line with the specific binding of both ^{11}Tyr-SS-14 and ^{125}I-sulpiride.

Correlation analyses

First, we performed univariate correlation analyses between the expression levels of the individual receptors (sst_2, sst_5 and D_2). This revealed a positive correlation between the expression of all three receptor subtypes; the correlation was especially high for sst_5 and D_2. Spearman (non-parametric) correlation coefficients were: $r = 0.384$ ($p=0.036$) for sst_2 and sst_5; $r = 0.377$ ($p=0.040$) for sst_2 and D_2; $r = 0.675$ ($p<0.001$) for sst_5 and D_2.

Subsequently, we analyzed the correlation between the expression levels of the individual receptors and the principal biochemical parameters of hypercortisolism (i.e. 24 hr UFC, mean diurnal serum cortisol and cortisol diurnal rhythm) and responses to the CRH-test and low-and high-dose dexamethasone suppression tests. None of these variables were significantly associated with the expression of any of the receptor subtypes. Moreover, after categorizing these nominal variables into tertiles to limit the effect of data heterogeneity, no significant associations could be identified.

DISCUSSION

This study investigated the (co-)expression of somatostatin and dopamine receptor subtypes in a series of 30 pure human corticotroph adenomas. We found that D_2 and sst_5 receptors are expressed in the majority of these adenomas and that co-expression of these receptor subtypes is a common phenomenon. Our data indicate that approximately 80% of corticotroph adenomas obtained from CD patients express one or more of these molecular targets, which could make them suitable candidates for D_2 and/or sst_5-targeted medical therapy.

In this series, we find that approximately 60% of CD patients have adenomas that significantly express sst_5 receptors. In a recent study, monotherapy with pasireotide (high sst_5 affinity) decreased urinary free cortisol (UFC) levels in 76% (22/29) of patients after 15 days of treatment (8). Complete normalization of UFC occurred in only 17% (5/29) of these patients (8). It needs to be emphasized, however, that this was a short trial and that the maximal cortisol lowering effects of pasireotide may require a longer duration of treatment in some patients. From this perspective, the percentage of complete responders found in this trial may still be an underestimation of the total number of responders with prolonged sst_5-directed therapy. On the other hand, different mechanisms of tac-

hyphylaxis have been described with the use of SS-analogues in the treatment of some, but not all neuro-endocrine tumours (17). Future studies will have to show whether this process of tachyphylaxis could affect the long-term efficacy of pasireotide therapy in human CD. An important safety concern with the use of this drug in CD is the deterioration of glucose tolerance, which is observed in approximately one third of patients (8).

The vast majority of adenomas, more than 80% of cases, clearly expressed D_2 receptors, which is similar to what has been found previously in another study by different analytical methods (4). These percentages are comparable, albeit somewhat higher than the percentage of initial responders (both complete and partial) observed in vivo with cabergoline monotherapy, which varies between 51 and 75% (4, 5, 18). In these studies, patients were treated considerably longer (3-6 months) than in the abovementioned 15-day pasireotide trial before response was assessed. Importantly, two of these studies also demonstrated that some patients on cabergoline monotherapy show a relapse after having been in full remission, which reduces long-term remission rates to 27-40% after approximately two years of treatment (5, 18). The mechanisms that cause these escapes from cabergoline monotherapy are not known. Receptor down-regulation or various post-receptor desensitization mechanisms may be involved in this process. Regardless of the cause, these treatment escapes pose a great challenge to physicians managing CD patients and limit the efficacy of cabergoline monotherapy as a long-term treatment option for human CD.

The observation that the majority of corticotroph adenomas in our study expressed both D_2 and sst_5 receptors suggests the possibility of combined targeting of these receptor subtypes. It has been shown that different sst and DA receptor subtypes can heterodimerize at the plasma membrane when they are activated simultaneously, leading to enhanced cAMP inhibition and functional synergism (9, 19). Moreover, co-activation of sst and DA receptor subtypes by chimeric dopastatin molecules may further enhance inhibition of hormone release by pituitary adenomas in vitro (20, 21). These studies all suggest potentially beneficial effects of combined sst_5 and D_2 targeting therapy, but clinical studies are needed to show whether this concept also translates into increased normalization rates in patients with CD.

An important issue from a clinical perspective is that we found a very low level of expression of sst_5 and D_2 in the corticotroph adenomas with invasive growth (Wilson grade III and IV). Whether this is due to loss of receptor expression in the course of dedifferentiation of these adenomas or whether low expression levels are causally related to extensive corticotroph adenoma growth, remains to be elucidated. Nonetheless, this finding could have clinical consequences, as not all subgroups of CD patients have the same likelihood

of receiving medical therapy at some point. It is well known that neurosurgical cure rates in patients with large corticotroph macroadenomas or those with invasive growth into structures surrounding the pituitary fossa, are decreased due to incomplete tumor removal (22). Therefore, these patients often require effective secondary therapies to achieve biochemical remission. Unfortunately, in the light of our findings, sst_5 and/or D_2-targeting agents are unlikely to be effective in this subgroup of patients.

It needs to be emphasized, however, that the group of corticotroph adenomas included in the present study can not be regarded as a representative sample for the total group of corticotroph adenomas as they would occur in clinical practice. Due to the problem of limited tissue availability in the case of corticotroph microadenomas, our data set contains a relatively large number of macroadenomas for which adenomatous tissue was more readily available. Moreover, a significant number of microadenomas that were initially included in this study, turned out to be a mixture of adenoma and surrounding normal pituitary tissue, as expressed by a GH/POMC ratio similar to that in normal anterior pituitaries and had to be excluded from further analysis. For that reason, our data set contains an overrepresentation of corticotroph adenomas in the higher Wilson classification grades (≥2). In clinical practice, it is estimated that macroadenomas and/or invasive corticotroph adenomas represent less than 10-20% of the total number of CD cases, whereas in our study 57% of cases belonged to this category (23, 24). Consequently, the true percentage of CD patients with grade 3 or 4 invasive adenomas and overall low sst/D_2 expression levels, will certainly be lower than the 23% identified in this study. In addition, it is important to note that microadenomas and noninvasive macroadenomas (grade 1 and 2 adenomas, respectively), which represent the vast majority of corticotroph adenomas, were found to have a very similar sst and DA receptor expression pattern. Therefore, our main findings in this study could be regarded as representative for both grade 1 and grade 2 corticotroph adenomas alike.

Previous *in vitro* studies have shown that glucocorticoids may directly down-regulate sst_2 expression in human and murine neuro-endocrine cell lines, whereas sst_5 is significantly less sensitive and D_2 is fully resistant to this type of glucocorticoid-induced down-regulation (25, 26). In this study we did not find a correlation between sst_2, sst_5 or D_2 expression levels and the height of the main biochemical parameters of hypercortisolism at the time of initial diagnosis, including 24-hour UFC and mean diurnal serum cortisol. The interpretation of the absence of these correlations is difficult, however, as most patients in our study were treated pre-operatively with cortisol-lowering drugs. Therefore, we do not know the exact effects of therapy-induced reductions in cortisol levels on the documented receptor expression levels in the adenomas. One can only hypothesize on the basis of the previous *in vitro* studies that reductions in cortisol are

likely to have resulted in a relative increase in sst_2 expression, rather than to have affected sst_5 or D_2 expression levels in these adenomas.

In conclusion, we demonstrate that the vast majority of human corticotroph adenomas express significant numbers of sst_5 and/or D_2 receptors, with co-expression of these receptor subtypes being a common phenomenon. These findings support the ongoing clinical studies with agents that target these receptor subtypes in patients with CD. Our results also suggest that combination therapy with both sst_5 and D_2 targeting agents could be a feasible next step, as this may increase the total number of CD patients that can be biochemically controlled by the use of these agents.

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Chapter 5

Early clinical experiences with combined somatostatin analogue, dopamine agonist and/or low-dose ketoconazole therapy in human Cushing's disease

Manuscript in preparation

INTRODUCTION

Recent studies show that complete normalization of urinary free cortisol levels occurs in approximately 40% of CD patients after 3-24 months of cabergoline monotherapy and in 17% of CD patients after 15 days of high-dose (2x600µg) pasireotide (SOM230) therapy (1-4). Whether or not the responders in these separate studies show a high degree of overlap or whether they constitute two different groups is not known. Either way, medical combination therapy with dopamine (DA) agonists and novel somatostatin (SS) analogues with high sst_5 affinity appears to be a feasible approach (5). In theory, SS-DA combination therapy could increase the total number of (complete) responders to this type of medical therapy. Hypothetically, it could also help in maintaining long-term remission rates by reducing the risk of tachyphylaxis, which can be observed under cabergoline monotherapy in human CD (2, 4). Moreover, *in vitro* studies using corticotroph tumour cells have shown that ketoconazole, which is used in many centers as monotherapy during the pre-operative phase to decrease cortisol levels, may display functional synergism with pasireotide in lowering ACTH secretion (Feelders et al., personal communication). Whether this also occurs in vivo has not been investigated until now.

For the above reasons, we are currently conducting a phase II open-label multi-center clinical trial to assess the efficacy and safety of a novel stepwise medical treatment approach with the use of the multiligand somatostatin analogue pasireotide (SOM230) with high sst_5 affinity in either a low-dose (3x100µg) or a higher dose (3x250µg), the dopamine D_2 agonist cabergoline and low-dose ketoconazole in patients with de novo or recurrent CD. The primary aim of this ongoing trial is to identify which percentage of CD patients can be biochemically normalized within a period of three months by the use of this treatment regimen. In this chapter we briefly describe four different patients that were included in this trial, who differ clearly in terms of disease presentation, adenoma characteristics and ultimate treatment outcome. The responses in these patients are discussed here to illustrate patient heterogeneity and as an extension of our *in vitro* findings in CD that were described in previous chapters. The complete details of this study and its results will be presented elsewhere.

CASE REPORTS

Patient 1

Patient 1 is a 42-year-old female with de novo CD due to a pituitary microadenoma (9 mm). Main complaints consisted of severe obesity (135 kg, BMI 44.5 kg/m2), uncon-

trolled hypertension and recently diagnosed type II diabetes mellitus. Urinary Free Cortisol (UFC) excretion was 5.1 fold the upper limit of normal (ULN) (figure 1). Pasireotide monotherapy did not significantly decrease UFC levels after 28 days of treatment (-6%). However, when the D_2 agonist cabergoline was added, her UFC values decreased sharply (-69%) to 1.6 fold the ULN at Day 56 of treatment. Finally, when low-dose ketoconazole (3 x 200µg) was added, UFC values normalized at Day 77. Over the study period, she reported a 5 kg weight loss, a spontaneous return of menses and diminished heat intolerance. In addition, her blood pressure had completely normalized and exercise capacity had increased. In this patient, however, we did observe an overt worsening of glucose tolerance, which required additional medical therapy.

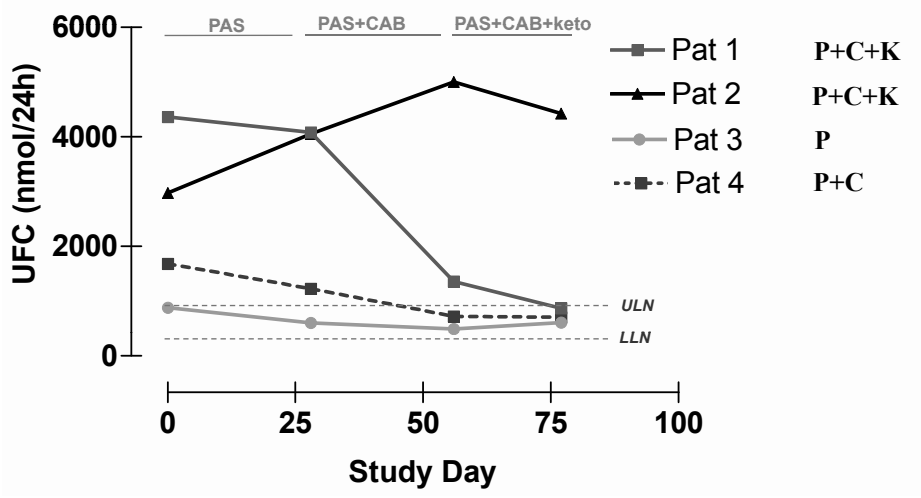

Figure 1: Urinary Free Cortisol excretion levels (in nmol/24 hr) in patients 1-4 enrolled in this study. Patients started at baseline (Day 0) with pasireotide monotherapy (Pas or P). If UFC was not normalized at Day 28, cabergoline (CAB or C) was added. If at Day 56 UFC was not normalized, low-dose ketoconazole (keto or K) was added until the study end at Day 77. ULN = Upper limit of normal for UFC assay. LLN = Lower limit of normal for UFC assay.

Patient 2

Patient 2 is a 67 year-old male with persistent CD after unsuccessful transsphenoidal adenomectomy one year prior to inclusion in this study. During that surgery, the 7 mm pituitary microadenoma had shown signs of invasive growth in the underlying sella; in fact, pathological examination of the biopsies from the surrounding sellar bone, showed intra-osseal (parasellar) remnant adenoma cells. Therefore, this adenoma was classified as Wilson Grade 3 (invasive microadenoma). At the study start, this patient complained of a mild centripetal weight gain (+5 kg), muscle weakness, easy bruising and fatigue.

At baseline, he was already receiving treatment for hypertension, osteoporosis and hypercholesterolemia. On pasireotide low and high-dose monotherapy, his UFC excretion did not decrease, but rather increased over time (figure 1). Also after the addition of cabergoline, UFC levels continued to rise. Only when low-dose ketoconazole was added, UFC excretion decreased slightly, but did not normalize. At the study end, the patient did not report any symptomatic improvements; neither did his blood pressure or laboratory values ameliorate.

Patient 3

Patient 3 is a 65-year old woman with de novo CD due to an invisible pituitary microadenoma, which was suggested by inferior sinus petrosus sampling (IPSS). Biochemical parameters of hypercortisolism were mildly elevated, but over the years had gradually resulted in increased abdominal size, 20 kg weight gain and progressive fatigue. On low-dose pasireotide monotherapy her UFC levels decreased slightly but did not normalize. On high-dose pasireotide therapy, however, UFC levels completely normalized and remained below the ULN throughout the entire study period (figure 1). She lost 3 kg in weight and reported some minor symptom improvements.

Patient 4

Patient 4 is a 43-year old woman with progressive symptoms of central obesity, easy bruising, hirsutism and amenorrhea. Her de novo CD was due to an invisible pituitary adenoma, which was proven by IPSS. Low- and high dose pasireotide monotherapy decreased her 24 hr UFC excretion slightly, but not sufficient (figure 1). After addition of cabergoline, however, UFC values completely normalized and remained in the mid-normal range until the end of the study period. She reported a marked improvement of her emotional state and exercise capacity, a spontaneous return of menses and a decrease in symptoms of hirsutism.

DISCUSSION

We here describe four patients that were included in an ongoing phase II, open-label multi-center clinical study that investigates the feasibility of a stepwise medical treatment regimen that combines the SS-analogue pasireotide with high sst_5 affinity, the D_2 agonist cabergoline and/or low-dose ketoconazole in patients with de novo or recurrent CD. As this is an ongoing clinical trial, final results of this study will be presented in detail elsewhere. However, these four patients are illustrative for the wide spectrum of treat-

ment responses, which can be observed with SS and/or DA-targeted medical therapy for this deleterious endocrine disease. Responses varied from partial or complete responsiveness to SS-analogue monotherapy or combined SS/DA analogue treatment, to a total lack of response to these analogues or their combination.

The reasons behind this large variability in treatment responses are multiple, but the most important factor appears to be tumor heterogeneity. As already described in chapter 4 of this thesis and also in previous publications, significant differences exist in somatostatin and dopamine receptor subtype expression levels in series of human corticotroph adenomas (1, 6, 7). Preferably, one would be able to correlate clinical outcome data with the corresponding *in vitro* data of adenomas of individual patients. In two out of four of the patients that we have described above, we were able to do so (patients 1 and 2).

Patient 1 had newly diagnosed CD when she entered the study and underwent transsphenoidal surgery approximately five months after completion of the study. In the time period between the study end and transsphenoidal surgery, she opted to continue the study drug regimen (extension study), which kept the UFC levels well within the normal range and further ameliorated her symptoms and signs of CD. When she underwent transsphenoidal surgery, we freshly isolated a part of the operative specimen and performed quantitative PCR for sst and DA receptor expression. The results are shown in figure 2. It shows she has clearly demonstrable sst_2, sst_5 and D_2 receptor expression, which is partially in line with her clinical response observed in the trial. The data are supportive of a D_2 mediated cabergoline effect, but do no fully explain why she did not respond better to sst-targeted monotherapy (pasireotide) in the first 28 days of the study.

Two issues need to be addressed in this respect. First of all, receptor status was assessed after 8 months of successful, glucocorticoid-lowering therapy in this patient. As has been described by many different studies and also in chapter 3 of this thesis, glucocorticoids can directly influence expression levels of sst in human neuro-endocrine cells, while for D_2 such an effect can not be demonstrated to the same extent or may even be absent (8-12). Especially sst_2 expression is sensitive for glucocorticoid-induced down-regulation and may be restored after correction of the hypercortisolism. Therefore, it is likely that the long-term normocortisolemia induced in this patient by the study drug regimen will have positively affected tumoral sst_2 expression levels in this patient. Therefore, measured sst_2 levels as shown in figure 1, may be an overestimation of tumoral sst_2 levels when the patient first entered the study. This may explain the absence of a therapeutic effect after 28 days of treatment by SS-multiligand pasireotide in this patient, even though sst_2 levels were well demonstrable after 8 months of therapy.

Another aspect could be the onset of rapid tachyphylaxis after the start of pasireotide monotherapy in patients with adenomas that are susceptible for such a phenomenon.

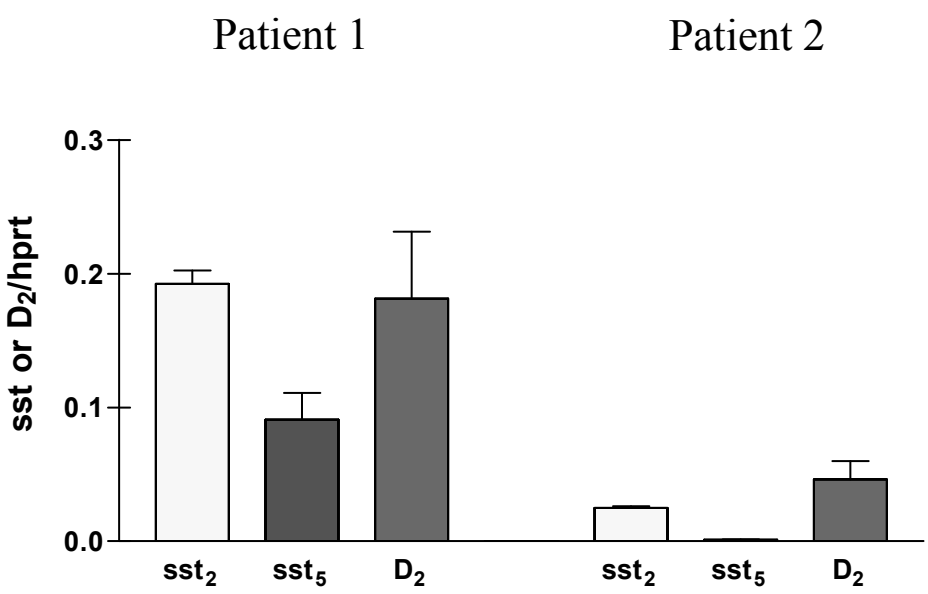

Figure 2: Sst_2, sst_5 and D_2 mRNA receptor expression levels in isolated corticotroph adenoma cells from patients 1 and 2, obtained at transsphenoidal adenomectomy. Values depict the mean (±SEM) of two measurements, assayed in duplicate. Expression levels are normalized against the housekeeping gene hprt.

If adenomas display high levels of sst_2 in the absence of sufficient levels of sst_5, desensitization to SS-analogues may occur. The *in vitro* susceptibility of sst_2 receptors for internalization and subsequent desensitization has recently been demonstrated by Liu and Cescato et al., while this was not demonstrable for sst_5 (13, 14). In fact, co-expression of sst_5 is believed to prevent sst_2 from rapid internalization and desensitization upon agonist exposure (15). When observed more in detail, our data show that in this patient after 10 days of low-dose pasireotide treatment, UFC levels dropped from 5.1 x ULN to 3.0 x ULN (-42%), but then rose steadily again to 4.8 x ULN (-6%, compared to baseline) on high-dose pasireotide therapy. This suggests a rapid desensitization mechanism upon SS-analogue therapy in this specific patient and may be associated with its high tumoral sst_2 expression levels. Whether this is a pattern of response that occurs more frequently in patients with CD, will have to be shown by final analyses on the total group of CD patients that participated in this study.

The other patient, for whom we had access to the surgically resected corticotroph adenoma tissue, was patient number 2. This patient had persistent CD after one, non-curative transsphenoidal surgery approximately 6 months before study entrance. As described before, UFC levels did not decrease in this patient with either pasireotide and/or cabergoline therapy. After the study end, when we retrospectively analyzed the archived adenoma tissue obtained during the first transsphenoidal surgery, it showed very low D_2 and sst_2 expression and total absence of sst_5 expression, which readily explains the com-

plete lack of response in this patient to either SS-analogue and/or DA-agonist therapy. This specific expression pattern is unusual for corticotroph adenomas in general, but may be observed more frequently in advanced corticotroph adenoma stages (stages III and IV, see chapter 4 of this thesis (16)). In fact, the adenoma of this patient had been classified as a stage III adenoma, due to its invasive growth in the underlying sella and the presence of intra-osseal adenoma cells in the parasellar bones.

In summary, we have here described four patients in an ongoing clinical trial, which exhibit different responses to SS-analogue and/or dopamine agonist therapy, which could be correlated at least partially to their adenoma sst and D_2 receptor status. Definitive results of this ongoing study, including correlation between treatment responses and in vitro receptor expression data, will be presented elsewhere.

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Chapter 6

Somatostatin receptor expression in a patient with Cushing's syndrome due to ectopic adrenocorticotropin secretion after successful mifepristone therapy

Submitted

ABSTRACT

Context: A 40-year old female patient presented in our clinic with Cushing's syndrome due to ectopic ACTH secretion of unknown primary origin. Cortisol levels decreased upon high-dose dexamethasone suppression testing, indicating intact tumor glucocorticoid responsiveness, while ^{111}In-pentreotide scintigraphy (OctreoScan) was negative. The patient was treated with the glucocorticoid receptor antagonist mifepristone, which dramatically improved her clinical symptoms. A repeat OctreoScan after 6 months mifepristone therapy showed the unequivocal presence of a bronchial carcinoid.

Objective: To combine in vivo and in vitro findings in a patient with ectopic ACTH-producing syndrome, in whom glucocorticoid suppression was still partially intact.

Methods: We obtained the fresh carcinoid tissue at surgery and investigated the expression of somatostatin and dopamine receptor subtypes in vitro by quantitative PCR, immunohistochemistry (IHC) and in vitro culturing of tumor cells.

In vitro results: The tumor was strongly positive for sst_2 and D_2 both at quantitative PCR and at IHC. Octreotide (sst_2 preferring analogue) and cabergoline (D_2-agonist) both decreased ACTH levels in the cultured tumor cells.

Conclusion: We describe a patient with an ACTH-producing bronchial carcinoid, in whom a direct down-regulatory effect of glucocorticoid levels on tumoral sst_2 receptor expression is suggested by a remarkable change in OctreoScan status after successful mifepristone therapy. Further studies will have to demonstrate whether glucocorticoid lowering or antagonizing therapy indeed improves the diagnostic yield of somatostatin receptor scintigraphy in patients with ectopic ACTH production of unknown primary origin in whom tumor glucocorticoid responsiveness is still (partially) intact.

INTRODUCTION

Ectopic adrenocorticotropin (ACTH) secretion (EAS) by a non-pituitary tumor is a rare cause of ACTH-dependent Cushing's syndrome (1). It is most frequently caused by either a bronchial carcinoid or a small-cell lung carcinoma, which accounts for approximately 50% of all cases, but also thymic carcinoids, gastro-entero-pancreatic neuro-endocrine tumors and medullary thyroid carcinoma are well-known causes of this syndrome (2, 3). The overall prognosis of the patient is largely determined by the nature of the underlying malignancy and the tumor stage at the time of diagnosis (1).

Primary therapy consists of surgical removal of the tumor, but in some EAS cases the primary tumor can not be identified by routine imaging procedures, including ultrasound, CT and MRI. The fact that a significant proportion of these tumors express somatostatin receptors (sst) allows the use of sst-scintigraphy with ^{111}In-pentreotide (OctreoScan) in the diagnostic work-up of these patients. The expression of sst is not only useful from a diagnostic perspective, it can also be used for therapeutic purposes because a significant number of EAS patients respond to traditional somatostatin (SS) analogues such as octreotide (1, 3-6). Apart from sst, dopamine receptors have also been proposed as potential targets in the medical treatment of these tumors (7).

However, a substantial number of tumors with EAS can not be detected with OctreoScan, although the exact percentage remains difficult to establish (6, 8-11). In addition, an important subset of EAS patients does not show any clinical or biochemical response to traditional SS-analogues that mainly target somatostatin receptor subtype 2 (sst_2) (1, 3). The reason for this large variability in functional sst_2 expression is not clear, but could be due to differences in glucocorticoid (GC) sensitivity of these tumors. It is well known that EAS tumors can differ strongly in their GC-responsiveness, which is reflected by the wide range of results obtained after high dose dexamethasone suppression testing in patients with EAS (12). At the same time, it also known that GCs can directly down-regulate sst expression in human neuro-endocrine tumor cells and more specifically sst_2 (13-15). Therefore, in the subset of EAS patients, in whom regulatory feedback by GC is still partially intact, cortisol-lowering or antagonizing therapy could directly influence tumoral sst_2 expression levels. If this occurs, one would expect to find evidence for such a mechanism both *in vivo* (serial OctreoScan imaging), as well as *in vitro* (analysis of the surgically resected tumor). We here present a case, for which we combined both investigations.

CLINICAL CASE

A female 40-year old patient was referred to us by her physician with a clinical suspicion of Cushing's syndrome. She complained of severe fatigue, which was progressive over the past few months, combined with easy bruising, muscle weakness and some mild alopecia. She had also noticed a weight gain of approximately 8 kg in the past months. She did not use any medication except for oral contraceptives. Previous medical history was unremarkable except for an extra-uterine pregnancy 15 years ago and a more recent episode of trigeminal neuralgia. At physical examination she had a Cushing-like appearance with marked facial and central obesity, combined with proximal muscle atrophy. Routine laboratory parameters were all within the normal range including serum potassium levels. Endocrinological laboratory evaluation revealed mildly elevated serum cortisol levels without any diurnal rhythm, insufficient overnight suppression of serum cortisol after administration of 1-mg oral dexamethasone and elevated 24-h urinary free cortisol (UFC) levels, see table 1. Plasma ACTH levels were within the high-normal range. A gadolinium-enhanced MRI of the pituitary did not reveal abnormalities. Bilateral inferior petrosal sinus sampling showed a basal central-to-peripheral ACTH gradient below 2.0 and after CRH administration below 3.0, indicating an ectopic cause of the ACTH-secretion. Results of an abdominal MRI were normal, but on a chest CT a small round nodule was seen in the right upper lung initially reported as an aspecific nodule (Figure 1A). Somatostatin recep-

Table 1. Laboratory parameters at time of diagnosis

	Value	Ref range
Serum cortisol (nmol/l)		
09.00 hr	792	200-800
17.00 hr	924	
22.00 hr	979	
24.00 hr	866	
Serum ACTH (pmol/l)		
09.00 hr	7.4	<11
17.00 hr	11.0	
22.00 hr	9.9	
24.00 hr	9.9	
1 mg dex. suppressiontest		
cortisol 09.00 hr (nmol/l)	646	< 50
high dose dex. suppression test		
(48 h 2mg/day)		
cortisol baseline (nmol/l)	530	
cortisol post-dex (nmol/l)	108	< 50

tor scintigraphy with ^{111}In-pentreotide (OctreoScan) did not show pathological uptake at that time (Figure 1B), nor did additional ^{18}F-DOPA-PET and ^{11}C-5-hydroxytryptamin-PET scans. The patient was treated with the glucocorticoid receptor antagonist mifepristone (RU-486, obtained from HRA Pharma, Paris, France) at a dose of 400 to 600 mg/day, which clearly improved the condition of the patient with 23 kg weight loss and disappearance of Cushingoid features. At follow-up, six months after initiating mifepristone treatment, a repeat chest CT showed the same nodule in the right upper lung without signs of anatomic growth (Figure 1C). However, at this time a repeat OctreoScan showed a positive uptake at the site of this nodule (Figure 1D and 1E). Subsequently, the patient underwent resection of the right upper lobe of the lung, which revealed a small tumor of 5 mm in diameter. Immediately after resection, the fresh carcinoid tissue was obtained for further analysis *in vitro* (see methods section). Pathological examination showed a neuroendocrine tumor with a positive staining for ACTH, synaptophysin and chromogranin A. The patient recovered well post-operatively without any major complications. She received hydrocortisone replacement therapy, which was gradually tapered. At present, 10 months after surgery, she is doing well without any evidence of a recurrence of hypercortisolism.

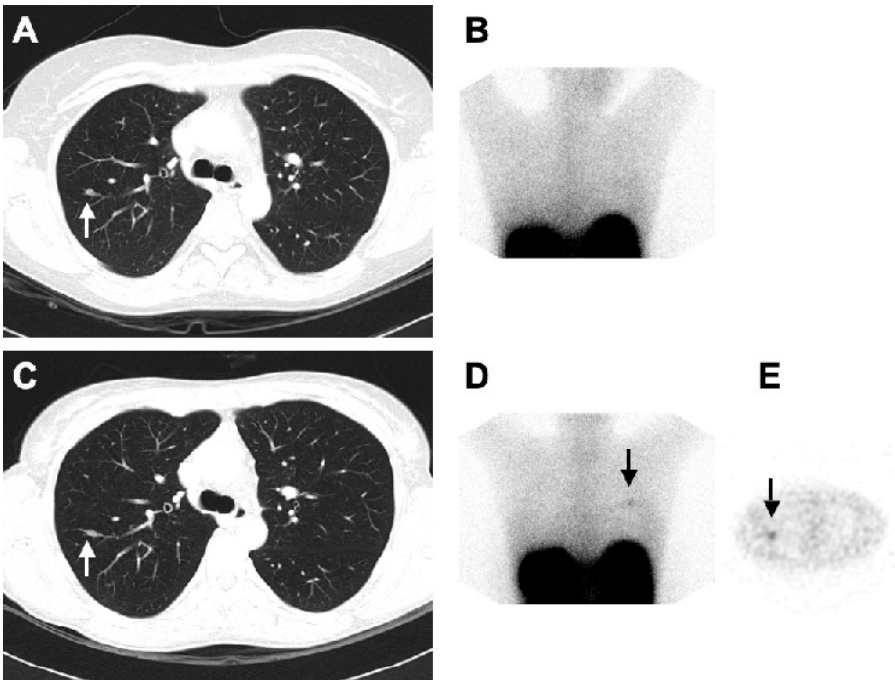

Figure 1: ^{111}In-pentreotide scintigraphy (OctreoScan) and CT imaging results in this patient before (A-B) and after 6 months of therapy with mifepristone (C-E). Before therapy was initiated, CT scan (A) shows a small round nodule in the right upper lung (white arrow), which is not visible at OctreoScan (B). After 6 months of therapy, the CT scan shows the same lesion (white arrow) within the upper lobe of the right lung (C). At that time a repeat OctreoScan shows pathological uptake at the site of the lesion (D and E; black arrows).

IN VITRO STUDIES

Informed consent

These studies were performed according to the rules of the ethics committee of our hospital. Informed consent was obtained from the patient.

Surgical tissue and cell isolation

After resection, the fresh carcinoid tissue was placed in 4˚C Minimal Essential Medium (MEM) with Earle's salts, supplemented with 10% fetal calf serum (FCS), L-glutamine (2 mmol/l), penicillin (10^5 U/l) and fungizone (0.25 mg/l). The tissue was dispersed with dispase 10^3 U/l (Roche, Almere, the Netherlands) + collagenase 2 mg/ml (Sigma Aldrich, Zwijndrecht, the Netherlands) at 37°C for 1 h to obtain a single cell population. Viable carcinoid cells were counted in a standard haematocytometer. 3 x 10^5 of the cells were used for qPCR analysis. The remainder of the cells were cultured in 48 well plates (Corning, Cambridge, USA) at a density of 40,000 cells/well for 4-6 days at 37 ˚C in a humidified incubator in 5% CO_2. At that time, culture media were refreshed and incubations were started with octreotide (Novartis, Basel, Switzerland), cabergoline (Pfizer, Capelle a/d Ijssel, the Netherlands) or their combination for 96 h. At the end of the incubation period, media were collected and stored at –20˚C for hormone analysis after addition of aprotinin (4 x 10^5 IU/ml medium; Bayer, Mijdrecht, the Netherlands) to prevent ACTH degradation. All experimental conditions were performed in quadruplicates.

Quantitative PCR

Quantitative PCR for the different SS and DA receptor subtypes was performed according to a protocol that has been described previously (13, 16). All samples were assayed in duplicate and values were normalized against the expression of the housekeeping gene hprt. Dilution curves were constructed to calculate PCR efficiencies (E) for every primer-probe set (17). Estimated copy numbers were calculated using the comparative threshold method with efficiency correction, as described previously (18).

Immunohistochemistry

The expression of sst_2 and D_2 in the carcinoid tissue was assessed by cutting cryostat sections (5µm) and performing immunohistochemistry according to a previously published method (19). The polyclonal anti-sst_2 antibody (Gramsch laboratories, Schwabshausen,

Germany) was used at a dilution of 1:2000 (overnight) and the monoclonal anti-D_2 antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) was used at a dilution of 1:400.

Statistics

All data were analyzed with GraphPad Prism software (San Diego, CA, USA). Data on hormone release are expressed as mean ± SEM. All experiments were run in quadruplicate. Overall differences between treatment groups were determined by ANOVA. In case of significant differences found by ANOVA, a multiple comparison between groups was performed with a Newman-Keuls test. P-values less than 0.05 were considered statistically significant.

IN VITRO RESULTS

The somatostatin and dopamine receptor mRNA subtype expression pattern in the carcinoid tumor cells is depicted in Figure 2. Sst_2 was highly expressed in this tumor (0.18 copies/hprt), while sst_1, sst_3 and sst_5 expression was low to very low (0.03, 0.01 and 0.03 copies/hprt, respectively). D_2 mRNA was also highly expressed in this tumor (2.13 copies/hprt).

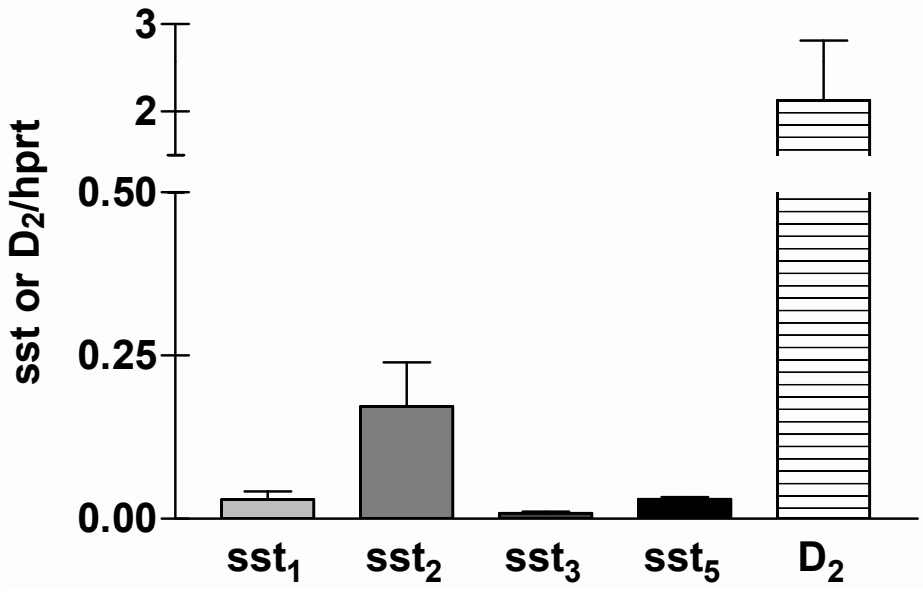

Figure 2: Somatostatin and dopamine receptor mRNA subtype expression. Values represent the mean ± SEM of two duplicate measurements. Expression levels are normalized against the housekeeping gene hprt.

In the cultured tumor cells of this patient, octreotide (OCT, -25%) and cabergoline (CAB, -25%) at 10 nM concentration both decreased ACTH release after 96 hr, although these inhibitions were not significant at the 0.05 significance level (Figure 3). The combination of OCT and CAB was less efficacious (-20%) than either agent alone.

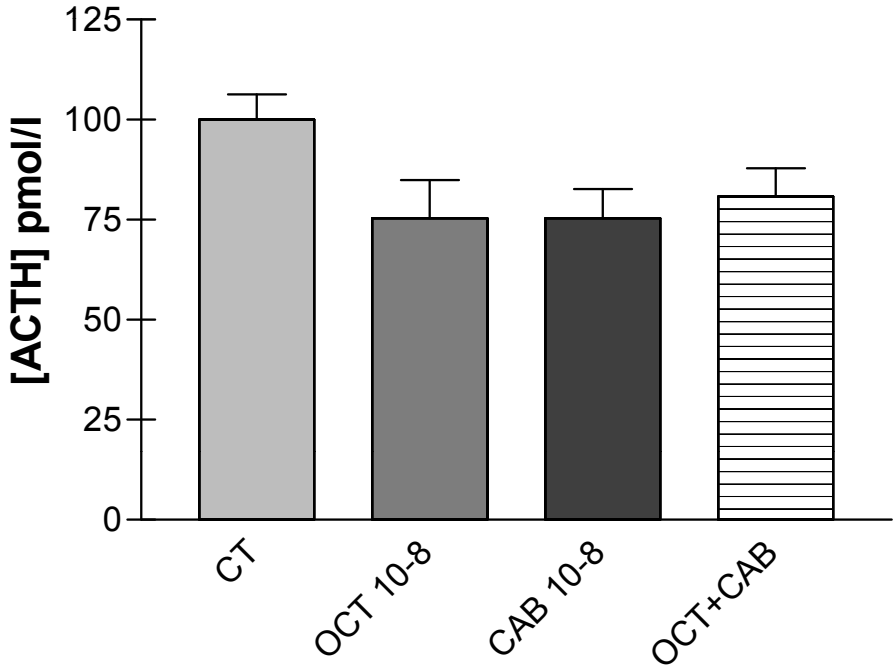

Figure 3: Inhibition of ACTH release by cultured carcinoid tumor cells of this patient after 96 hr. Cells were cultured in the absence (control, CT) or presence of octreotide 10^{-8} M (OCT), cabergoline 10^{-8} M (CAB) or their combination (OCT+CAB) in 10^{-8} M. At the end of the incubation time, media were collected and ACTH levels were determined. All experimental conditions were performed in quadruplicate. Values represent percent change ± SEM relative to control (= untreated cells).

At immunohistochemistry the tumor tissue was clearly positive for sst_2 with a membranous staining pattern, which was not present when the primary sst_2 antibody was pre-incubated with an immunizing sst_2 receptor peptide or with omission of the primary sst_2 antibody (Figure 4A-D). D_2 was also strongly expressed by this tumor (Figure 4E).

DISCUSSION

We here describe a patient with Cushing's syndrome due to an ACTH secreting bronchial carcinoid, in whom ^{111}In-pentreotide scintigraphy (OctreoScan) became positive after 6 months of successful GC antagonizing therapy with mifepristone. During that period,

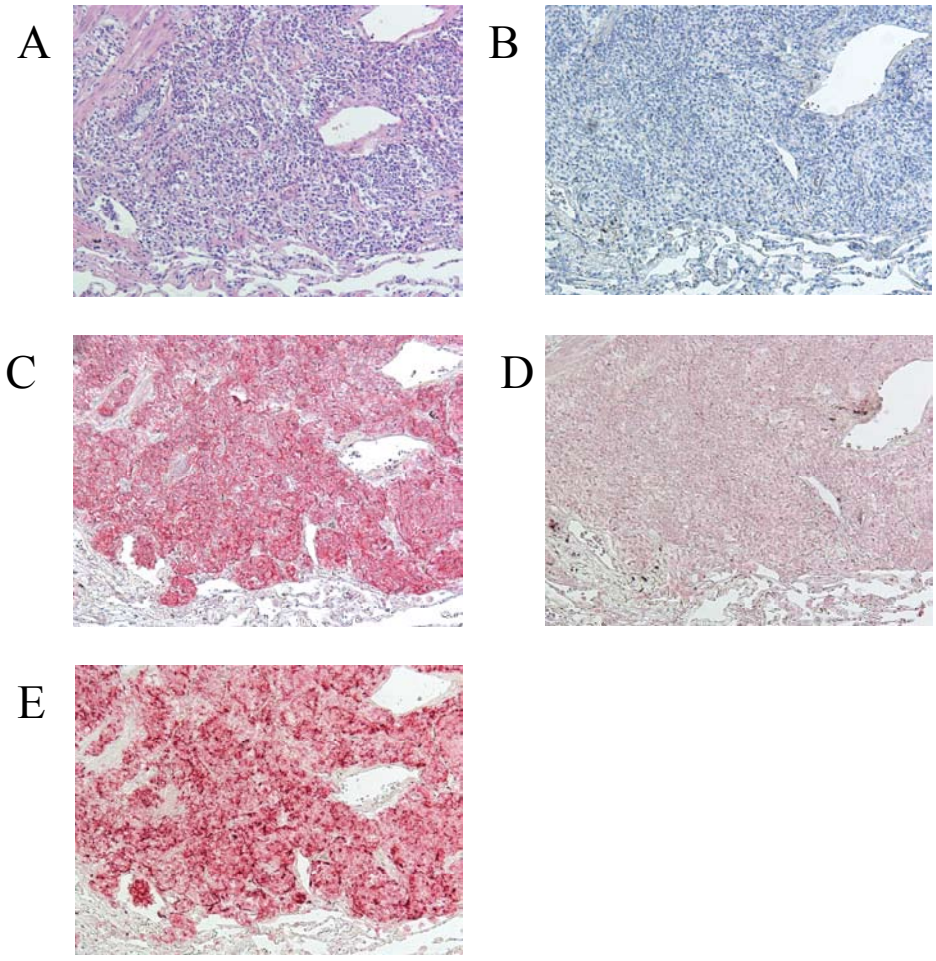

Figure 4, A-E: Immunohistochemistry for the sst_2 and D_2 receptor in the primary carcinoid tissue of this patient. All photographs were taken at a magnification of 100x. A: Hematoxylin and eosin stain. B: Negative control (omission of the primary sst_2 antibody). C: sst_2 polyclonal antibody. D: sst_2 antibody after immunoneutralization with an immunizing sst_2 receptor peptide. E: D_2 monoclonal antibody.

no anatomical growth of the lesion was observed on the accompanying CT scan, which could account for this change in OctreoScan status.

A sufficient degree of sst_2 expression is required to detect neuro-endocrine tumors via ^{111}In-pentreotide scintigraphy. Different studies have demonstrated that GC can directly down-regulate sst_2 expression in neuro-endocrine tumor cells (13-15, 20) and this may also explain the low levels of sst_2 in human corticotroph adenomas (21-23). In the human (pancreatic) neuroendocrine tumor cell line BON, we previously demonstrated that dexamethasone dose-dependently down-regulates sst_2 expression, which could completely be abrogated by co-incubation with mifepristone (13). For down-regulation

of sst_2, however, an intact GC signaling pathway needs to be present within these cells, which is not the case in many of the neuro-endocrine tumors that cause ectopic Cushing's syndrome (24, 25). In our patient, however, the glucocorticoid feedback system was still partially intact, as demonstrated by an absent suppression of serum cortisol on low-dose 1 mg DST, but a strong suppression on high-dose dexamethasone suppression testing (HDDST). This likely explains why after alleviation of the functional hypercortisolism by mifepristone therapy, sst_2 expression levels were restored.

The reported sensitivity of diagnostic OctreoScan in ectopic Cushing's syndrome is 50% at best in most series (1, 8, 11, 26, 27). It is important to note, however, that most of these data were obtained in patients with untreated ectopic Cushing's syndrome. As demonstrated by the results in our patient, the diagnostic accuracy of the OctreoScan may be increased after resolution of hypercortisolism in those EAS tumors, in which the GC signaling system is still (partially) intact. This is clinically relevant because in 12 to 18 % of patients the primary cause of EAS can not be localized initially (1), frequently necessitating a bilateral adrenalectomy. Future studies should prospectively investigate the diagnostic value of repeated OctreoScan after GC lowering or GC receptor antagonizing therapy.

The appearance of clearly demonstrable levels of sst_2 in our patient following mifepristone treatment strongly suggests the presence of a dynamic and potentially reversible relationship between GC action and the expression of sst_2 within neuro-endocrine tumors, including some cases of EAS. In this specific patient, we could not assess at what point of time the upregulation of sst_2 occurred as we only had access to two serial OctreoScans in a 6-month interval. An interesting question would further be whether this visible return of sst_2 expression also correlates with a response to treatment with SS analogues resulting in decreased ACTH production. If so, this could have implications for secondary treatment options in EAS patients that are not cured by surgery alone and in whom bilateral adrenalectomy is not feasible or desirable. Temporary pre-treatment with GC lowering (e.g. ketoconazole) or GC receptor antagonizing (mifepristone) therapy could result in a reappearance of sst_2 expression and consequently increase the efficacy of sst_2-preferring compounds such as Octreotide.

It is important to note that such a proposed mechanism of action only pertains to those cases of EAS, in which some form of GC regulation is still present. It is known that the majority of EAS cases will not suppress serum cortisol levels on HDDST (12). However, it is well conceivable that the minority of EAS cases, that do adequately suppress on HDDST, may constitute an important part of the group of EAS cases that are negative on OctreoScan.

In this tumor sst_2 was highly expressed: 0.18 copies/hprt. For comparison, we have measured sst_2 expression data recently in a large series of corticotroph adenomas with identical analytical methods. In these corticotroph adenomas the mean sst_2 expression level was 0.04/hprt (22). EAS tumors can display substantial numbers of sst_2 receptors, as demonstrated by the percentage of OctreoScan positive EAS tumors in several series (1, 3, 8, 11). These receptors can also be functional, as some EAS patients display a favourable response to Octreotide therapy in contrast to patients with CD (4, 28).

The fact that sst_2 expression levels tend to be higher in patients with EAS than in patients with CD is not surprising by itself. If EAS tumors are fully insensitive to glucocorticoid feedback, as demonstrated by absent or insufficient suppression at HDDST, then GC-induced down-regulation of sst_2 receptors is not likely to be present. We have investigated this phenomenon previously *in vitro*, in which the severely glucocorticoid-resistant EAS cell line DMS-79 did not show any signs of sst_2 down-regulation, even at the highest dexamethasone doses of 100 nM (13). As stated previously, the majority of EAS tumors will belong to this category, because in approximately 90% of EAS patients serum cortisol levels are not adequately (>50%) suppressed after HDDST (3). This does not pertain, however, to our patient, in whom HDDST did decrease serum cortisol levels (>50%) and in whom a negative OctreoScan was observed at the time of initial diagnosis. This indicates that some degree of intrinsic GC responsiveness was present in this tumor and that upon reversal of functional hypercortisolism by mifepristone therapy, sst_2 expression was restored. It is therefore important to note that the tumoral sst_2 levels we have measured *in vitro* in this individual patient after mifepristone therapy are not necessarily representative for the majority of fully GC resistant EAS tumors in clinical practice.

D_2 was also well expressed in this tumor (2.13 copies/hprt), which is in line with the reports by Pivonello et al. that these tumors can respond to cabergoline therapy, depending on their D_2 receptor status (7). The level of expression in this tumor was in the same order of magnitude as what we observed earlier for corticotroph adenomas (22). The fact that, in contrast to the sst_2 receptor, the expression levels between CD and EAS do not differ significantly for D_2, can be explained by the fact that the D_2 receptor gene does not appear to be as sensitive for GC regulation as sst_2 and to a much lesser degree sst_5 (13).

For both sst_2 and D_2, the mRNA expression levels were confirmed by the clear presence of sst_2 and D_2 at immunohistochemistry of sequential tumor tissue sections. Moreover, the expression data correlated with the inhibitory trend of Octreotide and Cabergoline in the primary cultured ACTH-producing carcinoid cells. The inhibition of ACTH release by cabergoline *in vitro* confirms earlier reports by Pivonello et al that these tumors can be responsive to D_2 agonists. In a study of six patients with EAS, 5/6 of these primary tu-

mors were D_2 receptor positive (7). Of these six patients, three were not cured by surgery and were treated adjuvantly with the D_2 agonist cabergoline, which induced a complete initial normalization of urinary free cortisol excretion in two of them. In another case report, combined treatment with the sst_2 preferring SS analogue Lanreotide and the dopamine D_2 receptor agonist Cabergoline induced complete remission in a patient with EAS due to a lung carcinoid (29). In our patient, we did not find signs of synergism between these receptors when they were co-activated *in vitro*.

In conclusion, we have described a patient with an ACTH-producing bronchial carcinoid tumor, in whom a direct regulatory effect of GC levels on tumoral sst_2 receptor expression is suggested by a remarkable change in OctreoScan status after successful mifepristone therapy. Further studies will have to demonstrate whether this is a more general mechanism of response in the subset of EAS cases, in which GC responsiveness is still partially intact.

ACKNOWLEDGEMENTS:

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Chapter 7

Functional characterization of the dopamine somatostatin chimeric molecule BIM-23A760

Submitted

ABSTRACT

Somatostatin (sst) and dopamine (D_2) receptor subtypes are co-expressed in various neuro-endocrine tumours and may show functional synergism. Novel dopamine-somatostatin chimeric molecules that bind to both receptor subtypes, have displayed superagonistic properties in earlier studies. Whether this is due to heterodimerization of target receptors or to enhanced activation of individual receptors, remains unknown. In this study we investigated the functional role of the different components of the novel dopamine-somatostatin chimera BIM-23A760 (high sst_2-D_2 affinity). We transiently transfected HEK-293 cells with D_2 and/or sst_2 cDNA and measured inhibition of forskolin-stimulated cAMP release by BIM-23A760 in comparison to its internal controls BIM-53097 (D_2) and BIM-23023 (sst_2). In D_2-monotransfected cells, BIM-23A760 was 15-fold more potent than BIM-53097 (EC_{50} 0.02 vs. 0.3 nM, $p<0.01$), even though both compounds have a similar D_2 receptor binding affinity (IC_{50} 15.9 and 22.1 nM, respectively). In contrast, in sst_2-monotransfected cells BIM-23A760 was 4-fold less potent than BIM-23023 (EC_{50} 0.04 vs. 0.01 nM, $p<0.01$), while BIM-23A760 sst_2 receptor binding affinity is 14-fold higher (IC_{50} 0.03 and 0.42 nM, respectively). In sst_2-D_2 co-transfected cells cAMP inhibition by BIM-23A760 was identical to that in D_2-monotransfected cells (EC_{50} 0.02 nM). Both the D_2-antagonist sulpiride and the sst_2-antagonist BIM-23454 were required to fully abrogate the effects of BIM-23A760. We conclude that in this transfected cell system, BIM-23A760 is a strong activator of sst_2, but also a remarkably potent activator of D_2 receptors, which cannot be explained by its binding affinity profile alone. Co-expression of sst_2 and D_2 does not enhance cAMP-inhibition by BIM-23A760 via either of these receptors. This suggests that the superagonistic properties of this compound in vivo, may at least partially be due to superior activation at the level of the individual (D_2) receptors.

INTRODUCTION

Dopamine (DA) and somatostatin (SS) receptors are (co-)expressed in many different neuro-endocrine tumours, including pituitary adenomas, gastro-entero-pancreatic tumours and carcinoids (1-3). Agents that target these receptors have been shown to be effective in inhibiting hormone release and/or tumour growth (4). Several years ago it was shown that dopamine receptor subtype 2 (D_2) and somatostatin receptor subtype 5 (sst_5), both members of the family of G-protein coupled receptors (GPCRs), could form heterodimers in a transfected cell system, leading to enhanced functionality of these receptors and synergism of action (5). Recently, similar phenomena have been described for the D_2 and sst_2 receptor (6). The discovery that these receptors can heterodimerize with a resulting increase in efficacy has opened up the way for potential combination therapy of both DA and SS-targeting agents in patients with neuro-endocrine tumours. Along with these developments, new compounds have been designed, which combine high-affinity binding to both DA and SS receptors within the same molecule, the so-called dopastatin chimeric molecules. In addition to the activation of both receptors individually, these compounds could in theory also draw both receptors together in a spatial manner, and thereby increase the degree of synergism between these receptors.

One of such chimeric compounds of interest is BIM-23A760. Saveanu and Jaquet have shown previously that this compound has superior efficacy in terms of GH-inhibition compared with traditional SS-analogues in some GH-secreting adenomas (7, 8). In a recent multicenter study, it was found that this compound also inhibited the proliferation rate of non-functioning pituitary adenomas (Florio et al. 2008 End. Rel. Ca.). Due to its binding affinity profile, with superior affinity for sst_2 and D_2, combined with a good affinity for sst_5, this compound could in theory also be of interest for the medical treatment of Cushing's disease (9), as well as the Ectopic-ACTH producing Syndrome (10, 11).

The mechanism behind the reported superpotency of these chimeric dopastatins has not been fully elucidated (1). In view of the clinical interest in this molecule and the ongoing debate on whether or not its superpotency can be attributed to heterodimerization of its target receptors, we performed the present study. With the use of an *in vitro* reporter gene assay, we aimed to study some of the functional characteristics of this interesting new dopastatin compound BIM-23A760, with the main research question being: can we ascribe the observed superpotency of this compound to heterodimerization or could it be due to superior activation of the individual receptors?

METHODS

Transfection studies and reporter gene assay

Since sst_2 and D_2 are both GPCR's that signal through the activation of cAMP, we investigated the functionality of BIM-23A760 in a reporter gene system, which indirectly measures intracellular cAMP levels. HEK293 cells (kind gift from Dr. A.P.N. Themmen, Erasmus MC) that natively do not express sst_2 and D_2, were transiently transfected with either human sst_2 cDNA (kind gift from Dr. Bell, Howard Hughes Medical Institute, Chicago, IL) or human D_2 cDNA (commercially available at UMR cDNA resource center; www.cDNA.com). For transfection, the calcium chloride precipitation method was chosen as this method has been used previously for this particular assay (12, 13). HEK-293 cells seeded at 20% confluency were transiently transfected with 2 µg of the cAMP-reporter plasmid pCRE6Lux (14), 2 µg pRSVlacZ (15) as a control for transfection efficiency, 2µg of human sst_2 cDNA and/or 0.1 µg of human D_2 cDNA and 14 µg carrier DNA in 75 cm^2 culture flasks (Costar, Cambridge, MA). Two days after transfection the cells were trypsinized and plated in 48-well tissue culture plates (Costar, Cambridge, MA). On the third day, medium was changed to serum-free medium supplemented with 0.1% bovine serum albumin. Cells were then stimulated with forskolin (10^{-6} M) for 6 hr in the presence or absence of the different BIM-agonists and/or antagonists. After 6 hr stimulation, cells were lysed with 25 mM Tris phosphate, pH 7.8, 8 mM MgCl, 1 mM dithiothreitol, 15% glycerol, 1% Triton X-100 and analyzed for luciferase (cAMP-responsive element driven) and *Renilla* luciferase (transfection control) activities using the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega Corporation, Madison, WI) (16).

Quantitative PCR

To check for adequate sst_2 and D_2 transfection efficiency, additional transfected cells were lysed for quantitative PCR (qPCR) in every experiment. This was performed via a previously described method (17). The sequences and final concentrations of the hprt, sst_2 and D_2 primer-probe pairs have been described previously (17, 18). The D_2 primer-probe set measures total D_2 expression (D_2 long + short isoform). All primers and probes were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). Samples were measured on an ABI Prism 7900 Sequence Detection System (Perkin Elmer, Foster City, CA) and normalized against the expression of the housekeeping gene hprt. All samples were assayed in duplicate.

Test substances

BIM-23A760, BIM-23023, BIM-23454 and BIM-53097 were provided by Biomeasure Incorporated (Milford, MA). Sulpiride was obtained from Sigma Aldrich (Zwijndrecht, the Netherlands). The binding affinities of the compounds are depicted in Table 1.

Statistical analysis

All statistical analyses were performed with GraphPad Prism version 3.02 software (San Diego, CA). EC_{50} values of dose-dependent inhibition of cAMP-release were calculated with non-linear regression curve fitting. Differences in dose-effect curves (EC_{50}) between the tested compounds were analyzed by the unpaired student t-test. Reported values are the mean of 2-4 different experiments. A significance level of 0.05 was set.

Table 1 Binding affinities (IC_{50}) in nM of compounds used in this study

Compound	Type	sst_1	sst_2	sst_3	sst_4	sst_5	D_2
BIM-23A760	agonist	622	0.03	160	>1000	42	15.9
BIM-23023	agonist	6616	0.42	87	2700	4.2	>1000
BIM-53097	agonist	n.d.	n.d.	n.d.	n.d.	n.d.	22.1
BIM-23454	antagonist	n.d.	32	n.d.	n.d.	n.d.	n.d.
Sulpiride	antagonist	n.d.	n.d.	n.d.	n.d.	n.d.	0.45

n.d. = not determined

RESULTS

Control experiments

To exclude the presence of natively expressed sst_2 or D_2 receptors in HEK-293 cells, lysates of mock transfected HEK cells were analyzed by quantitative PCR. No expression of sst_2 or D_2 was demonstrable. Moreover, neither the agonists BIM-23A760, BIM-23023 and BIM-53097, nor the antagonists BIM-23454 and sulpiride had an effect on forskolin-stimulated cAMP production in these mock-transfected HEK-293 cells (data not shown).

Sst_2 monotransfected state

In sst_2 monotransfected cells, the BIM-23A760 chimera induced a dose-dependent decrease in forskolin-induced cAMP production with an EC_{50} of 0.04 nM (figure 1). BIM-23A760 was 4-fold less potent, however, than its internal control BIM-23023 (EC_{50} 0.01 nM, $p<0.01$), even though BIM-23A760 has a 14-fold lower binding IC_{50} for the sst_2 recep-

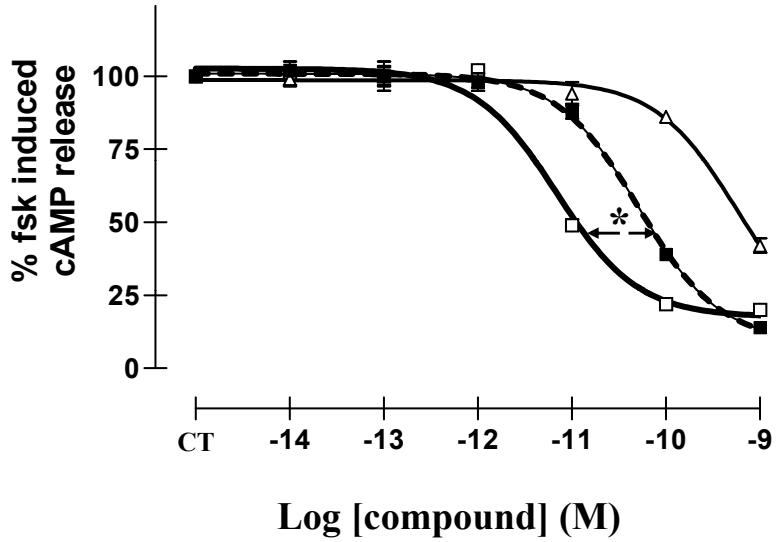

Figure 1: Sst_2 monotransfected cells. Forskolin-induced cAMP-inhibition by dopastatin BIM-23A760 (dashed line, solid squares), sst_2 agonist BIM-23023 (solid line, open squares) and BIM-23A760 in the presence of sst_2 antagonist BIM-23454 10^{-7} M (solid line with open triangles). The EC_{50} value of BIM-23A760 is shifted 4-fold to the right, compared to BIM-23023: 0.04 nM vs. 0.01 nM; * = p-value <0.01; CT = control. The values represent the mean ± SE from two different experiments, each performed in quadruplicates.

tor (0.03 nM vs. 0.42 nM, respectively, see Table 1). Maximum inhibitions were similar for both compounds (BIM-23A760: –86% vs. BIM-23023: –80%, p>0.05). The inhibitory actions of both BIM-23A760 and BIM-23023 could be antagonized by co-incubation with the sst_2 antagonist BIM-23454.

D_2 monotransfected state

In D_2 monotransfected cells, BIM-23A760 induced a dose-dependent decrease in forskolin-induced cAMP production with an EC_{50} of 0.02 nM (figure 2). In these cells, BIM-23A760 was 15-fold more potent than its internal control BIM-53097 (EC_{50} 0.30 nM, p<0.01), even though both compounds have a relatively similar binding IC_{50} for the D_2 receptor (15.9 and 22.1 nM, respectively). Maximum inhibitions were similar for both compounds (BIM-23A760: –88% vs. BIM-53097: –82%, p>0.05). Co-incubation with the D_2 antagonist sulpiride completely antagonized the inhibitory effects of both compounds.

Sst_2-D_2 cotransfected state

Sst_2 and D_2 cDNA were co-transfected in specific amounts, so that comparable mRNA copy numbers of these receptors were expressed: 0.66 (sst_2) and 0.58 (D_2) copies/hprt (figure 3).

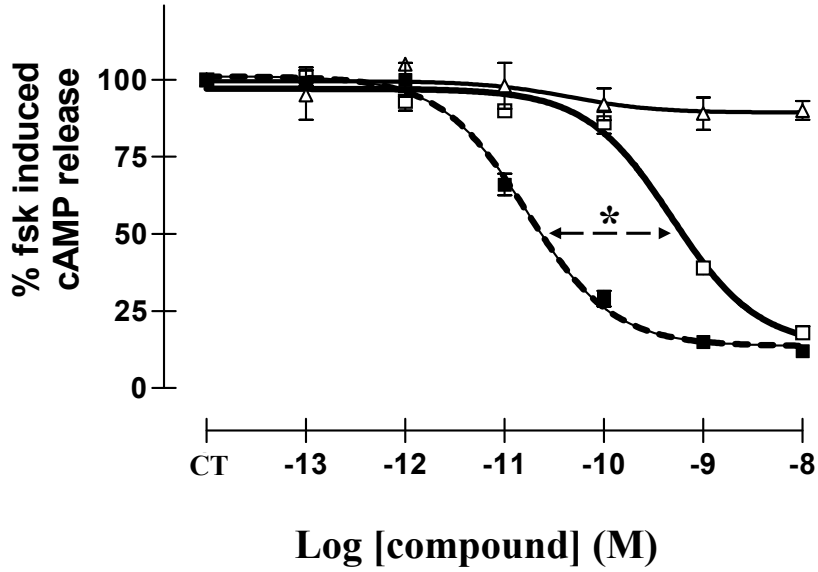

Figure 2: D_2 monotransfected cells. Forskolin-induced cAMP-inhibition by dopastatin BIM-23A760 (dashed line, solid squares), the D_2 agonist BIM-53097 (solid line, open squares) and BIM-23A760 in the presence of D_2 antagonist sulpiride 10^{-4}M (solid line with open triangles). The EC_{50} value of BIM-23A760 is shifted 15-fold to the left, compared to BIM-53097: 0.02 nM vs. 0.30 nM; * = p-value <0.01; CT = control. The values represent the mean ± SE from two different experiments, each performed in quadruplicates.

In this sst_2-D_2 cotransfected state, the monotargeting compounds BIM-23023 (sst_2) and BIM-53097 (D_2) had the same EC_{50} values as compared to their efficacy in the monotrans-

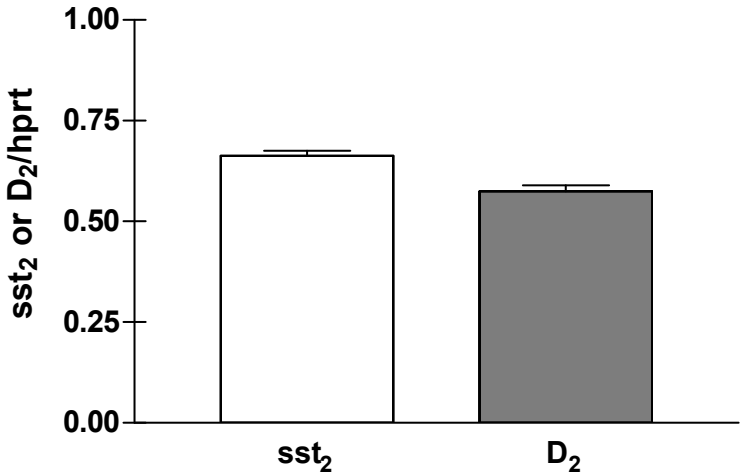

Figure 3: Sst_2-D_2 cotransfected cells. Sst_2 and D_2 mRNA expression levels as determined by quantitative RT-PCR. Values represent the mean ± SE of two independent experiments, assayed in duplicate.

fected states: BIM-23023 0.01 nM and BIM-53097 0.3 nM (figures 4A and 4B). The sst_2 and D_2 selective antagonists BIM-23454 and sulpiride were able to antagonize these effects.

In the sst_2-D_2 co-transfected state, BIM-23A760 displayed the same EC_{50} as shown previously for the D_2-monotransfected state: 0.02 nM (figure 4C). When only the sst_2 antagonist BIM-23454 was added, a strong dose-dependent decrease remained with an EC_{50} of 0.03 nM ($p>0.05$ for difference in EC_{50} values between BIM-23A760 and BIM-23-A760+BIM-23454). When only the D_2 antagonist sulpiride was added, a similarly strong dose-dependent decrease remained with an EC_{50} of 0.04 nM ($p>0.05$ for differences in EC_{50}), which was identical to the EC_{50} of BIM-23A760 in the sst_2 monotransfected state. However, when both antagonists were added, the inhibitory effects of BIM-23A760 were abrogated (EC_{50} 4.3 nM, $p<0.001$).

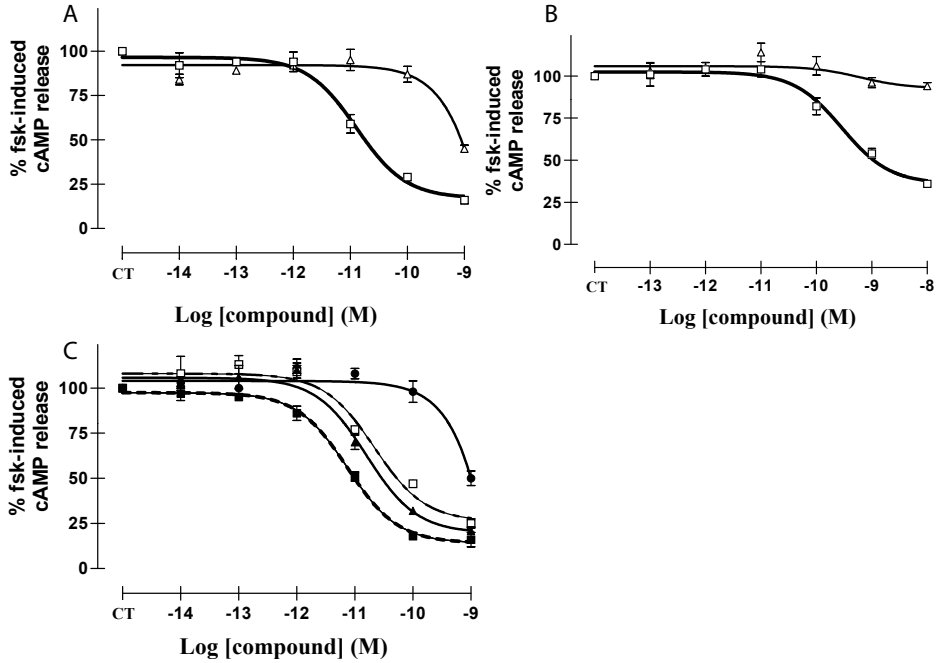

Figure 4 A-C: Sst_2-D_2 cotransfected cells. **A:** Forskolin-induced cAMP-inhibition by sst_2 agonist BIM-23023 in the absence (solid line, open squares) and presence of sst_2 antagonist BIM-23454 10^{-7}M (solid line, open triangles). **B:** Forskolin-induced cAMP-inhibition by D_2 agonist BIM-53097 in the absence (solid line, open squares) and presence of D_2 antagonist sulpiride 10^{-4}M (solid line with open triangles). **C**: Forskolin-induced cAMP-inhibition by dopastatin BIM-23A760 alone (dashed line, solid squares), BIM-23A760 + sulpiride (solid line with solid triangles), BIM-23A760 + BIM-23454 (dashed line, open squares) and BIM-23A760 + sulpiride + BIM-23454 (solid line, solid circles). CT = control. The values represent the mean ± SE from two different experiments, each performed in quadruplicates.

DISCUSSION

In this study we have investigated the functional role of the different components of the sst_2-D_2 chimeric molecule BIM-23A760, which determine its overall cAMP-inhibitory effect *in vitro*. We found that the activity of this compound via the D_2 receptor was higher than expected, whereas the activity via the sst_2 receptor was lower than expected on the basis of its reported D_2 and sst_2 binding affinities.

More in detail, we found that in D_2 transfected cells BIM-23A760 was 15-fold more potent than its internal control BIM-53097, even though the reported binding affinities of these compounds for the D_2 receptor are reported to be similar (15.9 and 22.1 nM, respectively). For the sst_2 receptor the exact opposite pattern was observed: BIM-23A760 was 4-fold less potent than its internal control BIM-23023, even though the binding IC_{50} of BIM-23A760 for the sst_2 is 14-fold higher than that of BIM-23023 (0.03 nM vs. 0.42 nM, respectively). It is important to emphasize that these observations were performed in sst_2 and D_2 monotransfected cell systems. In other words, the discrepancy between the observed efficacy *in vitro* and the reported binding affinity for both of these receptors, appears to be a direct feature of BIM-23A760 towards these individual sst_2 and D_2 receptors. Alterations in ligand binding characteristics by the simultaneous co-expression of the other sst_2 or D_2 receptor can not explain these observations.

The fact that dissociation is observed between binding affinities and *in vitro* efficacy, is not surprising by itself. It is well known that binding affinity (IC_{50}) is only one of many factors that ultimately determine the signal transducing effects of any given compound, including DA and SS analogues. This issue has been elaborately described by Dr. Schonbrunn in a recent review article and has been given the term "selective or biased agonism" (19). This refers to the phenomenon, by which different agonists that bind to the same receptor may trigger completely different post-receptor signalling pathways. Moreover, occupancy time of the receptor, internalization rates and recycling pathways may also vary considerably among different agonists of the same receptor (20).

One reason for our observation that the efficacy of BIM-23A760 via sst_2 is lower than expected, may be the susceptibility of sst_2 for internalization and degradation. Previous studies in transfected HEK-293 cells have shown that sst_2 rapidly internalizes upon agonist stimulation (20). Importantly, Cescato et al. have found that high-affinity binding to sst_2 is a prerequisite for an agonist to trigger the internalization process, also in HEK 293 cells (21). Moreover, they found that the agonist with the highest sst_2 binding affinity in their study, BIM-23244, also had the highest sst_2 internalization potency. Given the fact that the sst_2 binding IC_{50} of BIM-23A760 (0.03 nM) is even 10-fold lower than that of BIM-23244 (0.3 nM), it is to be expected that BIM-23A760 induced an even faster internalization of sst_2 receptors in our experiments, which may account for a relative decrease in efficacy. To our knowledge, similar internalization processes that are induced by high-affinity ligands

have not been described for the D_2 receptor. The question remains of course whether these processes, observed in (mono)transfected cell systems, occur in a similar fashion in vivo, where many different receptors are expressed in the close vicinity of these sst_2 and D_2 receptors. It has been shown for instance that sst_5 may directly decrease the internalization rate of sst_2 (22). This may prove to be a favourable determinant of the efficacy of BIM-23A760 in human neuro-endocrine tumors, which often co-express sst_2 and sst_5.

In the sst_2-D_2 co-transfected state, we had hypothesized that enhanced potency might occur because of synergism between the sst_2 and D_2 receptor. This has previously been shown to occur in a transfected cell system between the sst_5 and D_2 receptor (5). In this study by Rocheville et al. hetero-oligomerization of these receptor subtypes was proposed to lead to a sst_5-D_2 dimer with increased affinity for both sst_5 and D_2-targeting agonists and enhanced receptor activation. Recently, another group also showed that in co-transfected HEK-293 cells, sst_2 and D_2 receptors form heterodimers upon activation by selective ligands, leading to enhanced D_2 signalling and also prolonged sst_2 internalization (6). In our study, however, BIM-23A760 displayed the same EC_{50} in the sst_2-D_2 co-transfected state as compared to the D_2-monotransfected state (0.02 nM), without any increase or decrease in IC_{50} due to the presence of the extra sst_2 receptors.

As said, the mono-targeting compounds had the same EC_{50} and EC_{max} values in the monotransfected compared to the co-transfected states. In other words, the abundant presence of additional sst_2 or D_2 receptors did not stimulate nor decrease the signalling via these receptors. This is different from what has been reported previously by some but not all research groups in the same field of study. Both Ren et al. and Saveanu et al. found that the chimeric molecule BIM-23A387 (high sst_2-high D_2 affinity) showed enhanced inhibitory effects in human GH-producing adenomas compared to incubation with the individual targeting compounds (23, 24). Ren et al., however, found that by the use of the D_2 antagonist sulpiride, all inhibitory properties of BIM-23A387 were lost, suggesting a functional interaction between sst_2 and D_2, whereas Saveanu et al. showed only partial loss of inhibition by BIM-23A387 in this setting. Saveanu et al. therefore conclude that the potent effects of BIM-23A387 can not be explained by the process of receptor heterodimerization alone, but may also depend on specific interactions between the chimeric molecule and the individual sst_2 and D_2 receptors (24).

In summary, we find that in this transfected cell system BIM-23A760 is a strong activator of sst_2, but also a remarkably potent activator of D_2 receptors, which can not be explained by its binding affinity profile alone. In our study, co-expression of sst_2 and D_2 does not enhance the cAMP-inhibiting capacities of BIM-23A760 via either of these receptors. This suggests that the reported superagonistic properties of this compound in vivo, may at least partially be due to superior activation at the level of the individual (D_2) receptors.

ACKNOWLEDGEMENTS:

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Chapter 8

General discussion

The principal aim of this thesis was to further explore the possibilities for a medical treatment of human Cushing's disease based on the use of somatostatin analogues and/or dopamine agonists.

Despite all advancements in surgical technique, selective transsphenoidal adenomectomy still fails to induce long-term cure in approximately 30% of patients with CD, even in most experienced hands (1). The group of patients with persistent or recurrent CD is known to suffer from increased morbidity and mortality and represents one of the most difficult clinical challenges for endocrinologists (2). Until now, medical therapy has only played a minor role in the treatment of these refractory patients (3-5). Antiserotinergic drugs have been employed in the past without much success (5). Retinoic acid derivatives, despite promising results *in vitro* and in some animal models, have failed to make the transition to clinical studies in human subjects (6, 7). Moreover, peroxisome-proliferator-activated-receptor-gamma agonists have not shown any significant efficacy in several recent clinical studies in humans with CD (8-10). The use of dopamine agonists including bromocriptine and cabergoline has been widely studied in CD, but its success is limited by the occurrence of treatment escapes in a subset of patients (3, 11). Therefore, the search for an effective and safe medical treatment for human CD continues. In this thesis we have focussed on the role of dopamine and somatostatin receptor subtypes as potential targets for such a medical therapy.

ANIMAL MODELS FOR CD

One of the most important rate limiting factors in the advancements of research into the origins and treatment of human CD, has been the rarity of the disease. Interestingly, in dogs this disease is much more common, perhaps over 1000-fold more (12-14). The fact that at Utrecht University dogs with CD can be treated by a therapeutic hypophysectomy, provided a more than welcome opportunity to increase the availability of corticotroph adenoma tissue for *in vitro* functional studies. In *chapter 2* we have described the characterization of 13 of these adenomas for the presence of the receptor subtypes, in which we were primarily interested from a human point of view: sst_2, sst_5 and D_2. We found that these adenomas do express all of these receptor subtypes, but in very different ratios than anticipated beforehand. Whereas in human CD sst_2 only plays a minor role, it appears to be the dominant receptor subtype in canine CD, both in terms of expression levels, as well as functional responses *in vitro*. At the same time, sst_5 only has a minor role in canine CD, while D_2 also has lower expression levels than what is observed in humans. Inter-species differences in glucocorticoid regulation of the expression of these receptor subtypes are likely to contribute to these differences between human and canine CD:

whereas glucocorticoids decrease sst_2 expression levels in humans, they remarkably increase the expression of sst_2 in canine corticotroph adenomas.

We do believe, however, that valuable research hypotheses can still be generated from the use of canine CD as a model for human CD. Due to the predominance of sst_2 and D_2 in canine CD, the use of analogues that target these specific receptor subtypes could be of interest. This could be performed by co-administration of an sst_2 (octreotide or lanreotide) and a D_2 (cabergoline) targeting drug. The advantage of the use of these compounds is that both have been proven to be effective in dogs for the treatment of insulinomas and reproductive disorders, respectively (15, 16) . In other words, they are known to bind to canine sst_2 and D_2 receptors with sufficient affinity and can induce clinical effects. Their combined *in vivo* use in canine CD could provide firsthand evidence for the possible functional synergism between sst_2 and D_2 receptors, which has been suggested by recent *in vitro* studies (17). Moreover, the use of the novel dopamine-somatostatin chimera BIM-23A760, which is also discussed in *chapter 7*, could be of great interest here. This compound has a very high sst_2 and D_2 binding affinity and is therefore likely to induce profound ACTH suppression in canine corticotroph adenomas, providing that the reported binding affinity profile of this compound is similar in humans and dogs.

Finally, it is necessary to emphasize that canine corticotroph adenoma tissue can be valuable for various research questions that address the different etiological aspects of CD. For example, one study used canine corticotroph adenomas to investigate novel molecular aspects of glucocorticoid receptor function in CD (18). This study would have been much more difficult to perform if only human corticotroph tissue was available.

HUMAN CORTICOTROPH ADENOMAS

An important aim of this thesis was to increase our understanding of how many patients with CD have a reasonable chance of ultimately responding to somatostatin and/or dopamine targeted medical therapy. Data on this topic were still scarce at the start of this study, being derived from relatively small series of patients. Moreover, it was not known to which extent sst_5 and D_2 were co-expressed within the same corticotroph tumors. We aimed to reinvestigate the distribution of these target receptor subtypes in a larger population of CD patients, as the presence of demonstrable receptors is likely to be the first and foremost predictor of a successful response to this type of therapy. The importance of sufficient receptor numbers has been demonstrated by several studies, in which a direct relationship was found between the expression levels of sst and DA receptor subtypes in GH and mixed GH/PRL-secreting pituitary adenomas and specific agonist

action, both *in vitro* and *in vivo* (19-22). The *in vitro* correlation has been demonstrated both at the mRNA level (21) as well as at the protein level by immunohistochemistry (19, 20).

The main results of our study in 30 human corticotroph adenomas are described in *chapter 4*. We found a large variation in expression levels of sst_2, sst_5 and D_2 in our series of adenomas, similarly to what has been described before in GH-producing and non-functioning pituitary adenomas (21, 23). Overall, we believe that the results from this study are encouraging, as we found that over 80% of patients express at least one of these target receptors at a level, where protein expression can be visualized by means of receptor autoradiography. Of course, demonstrable protein expression at receptor autoradiography does not necessarily equal biological response, but at least the molecular substrate can be demonstrated on the cell membrane of the corticotroph adenoma cells. Various other factors, apart from the number of receptors, will ultimately determine how many patients will show a clinical response to sst_2/sst_5/D_2 targeted therapy. In prolactinomas, for instance, loss of the Gi2 alpha protein can cause resistance to the dopamine agonist bromocriptine, even though sufficient D_2 receptors are present on the cell membrane (24). In different neuro-endocrine tumors, especially carcinoids, various mechanisms of tachyphylaxis have been described (25). New studies will have to investigate whether similar phenomena also affect response rates in corticotroph adenomas.

THE ROLE OF GLUCOCORTICOIDS

One aspect that could play a decisive role in the efficacy of sst/DA-targeting agents at any given time during therapy, is the level of circulating glucocorticoids. Before the start of this thesis it had already been clearly demonstrated that murine sst_2 gene expression was under direct control by glucocorticoids, while sst_5 appeared to be less sensitive for this type of regulation (26, 27). For D_2, not much was known on glucocorticoid regulation in human neuro-endocrine tissues.

In *chapter 3* we have described the results of our study on glucocorticoid regulation of sst and DA receptors in three different human neuro-endocrine cell lines. Our results confirm the previous studies in murine corticotroph cells that sst_2 is highly sensitive, while sst_5 is far less sensitive for this type of down-regulation. We also found that the D_2 gene is completely insensitive for down-regulation by high levels of glucocorticoids. This could be an interesting finding for future medical therapies of CD. It may well explain why D_2 receptors are so highly expressed in the majority (80%) of corticotroph

adenomas, while expression levels of especially sst_2 are much lower *(chapter 4)*. We also found that glucocorticoid-induced down-regulation is a dynamic and also reversible process, when glucocorticoid levels fall. This could be an important observation from a clinical perspective, as this means that the induction of an initial biochemical remission in a patient with CD may aid to increase the total number of pharmaceutical targets in the adenoma tissue, including sst_2 (see figure 1).

In the same study we found that in the cell line derived from an ectopic-ACTH producing neuro-endocrine tumour (DMS-79), all receptors (including sst_2 and sst_5) were insensitive to high levels of glucocorticoids. We found this an interesting observation, as this first of all confirms the clinical experience that many tumours that cause the ectopic ACTH-producing syndrome (EAS) are positive on a diagnostic OctreoScan, which primarily identifies the presence of sst_2 receptors (28). In other words, these neuro-endocrine tumours express high levels of sst_2 receptors, despite the continuous hypercortisolemic environment they are exposed to. Gross mutations in the glucocorticoid receptor gene have been described in these tumours, which could explain the resistance of these tumours to glucocorticoid-mediated down-regulation of sst_2 receptors (29). However,

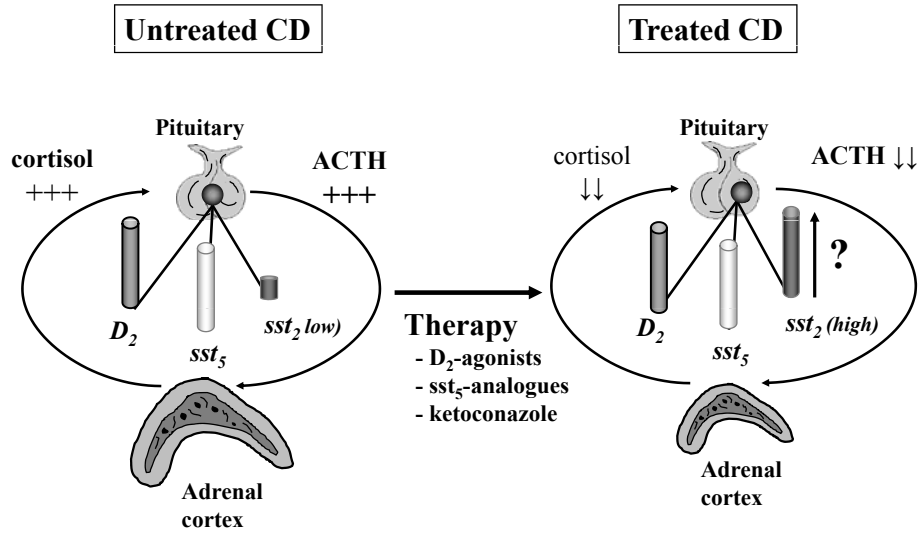

Figure 1: Hypothetical scheme of the effect of glucocorticoids on sst_2, sst_5 and D_2 expression in a corticotroph adenoma. In untreated CD (left panel), ACTH overproduction leads to chronic hypercortisolism, which down-regulates sst_2, but not sst_5 or D_2 expression. In treated CD (right panel), e.g. by sst_5 or D_2-directed medical therapy, alone or in combination with ketoconazole, lowering of ACTH levels reduces endogenous hypercortisolism and relieves the hypercortisolistic pressure on the pituitary, which in turn could lead to an in vivo reappearance of sst_2 expression. The latter would result in regained efficacy of sst_2-preferring compounds and thus enhance the pharmacological options for medical therapy. Adapted from (41).

not all EAS tumours are positive on Octreoscan (30, 31). Our hypothesis was that in this group of EAS tumours, apparently some degree of glucocorticoid regulation would be residually active. In these patients, reduction of circulating cortisol levels, could consequently lead to a reappearance of sst_2 expression, if this regulatory system is still intact.

Recently, we identified a patient in our clinic that suffered from EAS due to a lung carcinoid. Her case history and additional *in vitro* studies are described in *chapter 6*. At the time of diagnosis, she had a negative OctreoScan, but a small pulmonary lesion on a regular chest-CT. We treated her for 6 months with the glucocorticoid receptor antagonist mifepristone, thereby inducing a pseudo-hypocortisolemic state. We refer to it as "pseudo"-hypocortisolemic, since measured glucocorticoid levels were high, but functionally inactive because of the glucocorticoid receptor blockade by mifepristone. When a chest-CT and OctreoScan were repeated after 6 months of therapy, the pulmonary lesion had not increased in size but did show increased (pathological) uptake, indicating a strong upregulation of sst_2 in this tumour, which provided important clinical evidence for a direct and dynamic relationship between sst receptor expression and *in vivo* glucocorticoid levels. When this pulmonary lesion was surgically resected in this patient, sst_2 receptors were also clearly demonstrable by quantitative PCR and immunohistochemistry. Finally, the functionality of these receptors was proven by the inhibitory effects of octreotide *in vitro* on ACTH release. Interestingly, this carcinoid tumour also expressed high levels of D_2 receptors, which is in agreement with findings from a previous study in EAS (32) .

The results from this case study suggest that in a minority of EAS tumors, in which partial GC-responsiveness is present, glucocorticoid lowering or antagonizing therapy may be used for two purposes. First of all, in patients with EAS in whom no tumour can be visualized, glucocorticoid lowering therapy may induce expression of sst_2 in these tumours and allow for their proper localization, after which curative surgery may be performed. Secondly, if complete surgical removal of the tumour is not an option, cortisol-lowering therapy increases the level of sst expression and may therefore sensitize the tumour for subsequent SS-analogue therapy. The clinical utility of such an approach requires further investigation, however.

CLINICAL ASPECTS OF NOVEL SST/DA-TARGETING THERAPY IN CD

In *chapter 4* we reported that the majority of patients with CD possess at least one molecular target (sst_5 or/D_2) in their corticotroph adenoma tissue for a possible therapeutic effect to occur. For that reason we designed a phase II open-label clinical trial in which a

stepwise medical therapy, consisting of pasireotide ± cabergoline ± low-dose ketoconazole, was evaluated for its efficacy in patients with de novo or recurrent CD. At present, patient enrolment for this trial has just been completed and final data analyses are currently ongoing. As the definitive results of this trial will be published elsewhere, it is not possible to discuss the results of this trial in full detail within the context of this thesis. In *chapter 5*, however, we present and discuss four illustrative cases for the different types of clinical responses we have observed during this trial. On a general note, we can state that the study has produced firm clinical evidence for the efficacy of combined sst_5 and/or D_2-targeted medical therapy in human CD. A few important issues, however, require further discussion as they could prove to be of substantial clinical importance.

Dose

In this trial we observed that already on low-dose pasireotide (3 x 100 µg), selected patients can respond with a dramatic reduction in serum and urinary cortisol levels. These reductions can be observed within days after the first administration of the study drug. This is an interesting observation, as we initiated treatment in these patients with only a quarter of the pasireotide dose that Boscaro et al. used in their study (total daily dose: 300 µg vs. 1200 µg)(33). The significant response on these low doses may be explained by the subnanomolar affinity of pasireotide for sst_5 (0.38 nM). For comparison, octreotide can induce significant clinical responses in the 100µg dose in acromegalic patients, while its affinity for sst_2 is around 1.0 nM. Therefore, from a pharmacological perspective, the 100 µg pasireotide dose should be able to induce similar biological effects in corticotroph cells, provided that the adenoma expresses relatively high levels of sst_5 receptors, which some patients clearly do (*chapter 4*). The occurrence of these rapid reductions in serum cortisol after the initiation of pasireotide therapy also has clinical implications. The occurrence of acute glucocorticoid withdrawal symptoms or even an overt Addisonian crisis is a realistic possibility during this type of treatment. Therefore, close follow-up of these patients needs to be provided upon treatment initiation.

Effects on glucose metabolism

Another important observation in this trial was that glucose homeostasis deteriorated significantly in two of the patients that we describe in *chapter 5*. This problem has also been clearly reported in the first study on pasireotide use in patients with CD by Boscaro et al., where over one third of patients had significant worsening of glycaemic control (33). Different biological mechanisms could be responsible for this phenomenon. First of all, it could be due to a direct effect of pasireotide on pancreatic insulin release. Sst_1, sst_2 and sst_5 receptors, which are all targeted by pasireotide, are well expressed in human

pancreatic islet cells and activation of these receptors, especially sst_2, has been shown to strongly decrease pancreatic insulin secretion *in vitro* (34). One argument that strongly rules against such a direct effect via inhibition of insulin release, however, are the observations by Van der Hoek et al. in acromegalic patients, in whom pasireotide administration acutely increased blood glucose levels without a concomitant major decrease in insulin levels (35, 36). The latter would rather suggest that the observed hyperglycemia is due to acute peripheral insulin resistance, presumably at the level of the liver, skeletal muscle or adipose tissue. Another possible cause for the acute hyperglycemia after pasireotide exposure could be located at a more central level, for instance in the arcuate nucleus. It is known that this structure in the central nervous system is important for glucose homeostasis and that somatostatin receptors have been demonstrated here, including sst_1 and sst_2 (37, 38). Since pasireotide has nanomolar binding affinity for both of these receptor subtypes, this may be a potential mechanism of action, which has been underinvestigated thus far. In conclusion, the mechanism of action of pasireotide-induced hyperglycemia remains poorly understood and strongly demands further investigation.

Effects on GH/IGF-1 axis

Another potential problem might be any direct effects of pasireotide therapy on GH and IGF-1 levels in CD patients. It is well known that pasireotide, via sst_2/sst_5 receptor activation, directly decreases GH release and hence IGF-1 levels in patients with acromegaly. Whether this also occurs in normal subjects without GH/IGF-1 axis overactivation, has not been reported. Normal somatotrophs, however, express sst_5 in significant numbers and in normal rats, primates and dogs pasireotide has been shown to significantly decrease GH and IGF-1 levels (39). In patients with CD, sustained hypercortisolism by itself causes a state of relative growth-hormone deficiency and therefore these patients may be at greater risk to become GH-deficient. Current and future clinical studies with pasireotide in CD patients should therefore include careful investigation of the effects on the GH/IGF-1 axis.

FUTURE DIRECTIONS: NOVEL DOPASTATIN CHIMERIC MOLECULES

Finally, in *chapter 7* we have investigated some functional aspects of the novel dopastatin chimera BIM-23A760. This compound has been postulated as a promising new compound for the future treatment of neuro-endocrine tumours, due to its unique binding affinity profile (IC_{50}): high D_2 (15.9 nM) and extremely high sst_2 (0.03 nM) affinity. Given the abundant (co-)expression of both of these receptors in various neuro-endocrine tumours and the fact that these receptors can heterodimerize *in vitro* (40), which may

result in enhanced combined potency, this compound is of great interest from a clinical perspective. Some earlier studies have already shown superagonistic properties of this drug in selected cases (23).

In our *in vitro* study, in which we transiently (co-)transfected sst_2 and D_2 cDNA into HEK293 cells and used a luciferase reporter gene system to determine efficacy of the different SS/DA compounds, we found that BIM-23A760 is an unexpectedly strong activator of D_2 receptors *in vitro*, while sst_2 activation is less pronounced than would be expected on the basis of its extremely high sst_2 binding affinity. Both receptors are functionally active, however, in the low nanomolar range. We did not find direct evidence for an enhanced effect by combined targeting of both sst_2 and D_2 receptors by BIM-23A760. Moreover, in the presence of selective sst_2 and D_2 antagonists, BIM-23A760 potently inhibits cAMP formation by either one of the receptors.

In our view, these results indicate that the superior effects of BIM-23A760 are at least partially due to individual receptor-ligand interactions, as opposed to mere heterodimerization. This enhanced activation of individual receptors can be due to many different factors that are involved in the process of ligand binding, receptor occupancy time, internalization rates and post-receptor processing of the ligand-receptor complex. At present, clinical studies in different phases (I-III) are ongoing with BIM-23A760 in human subjects and results of these studies are eagerly awaited. One crucial aspect for the future applicability of this drug will be whether BIM-23A760 will provide superior efficacy, compared to combined SS-analogue and DA-agonist therapy.

In conclusion, we find in this thesis that the majority of patients with CD possess a molecular target for sst_5 or D_2 directed medical therapy, which makes them candidates for therapy with either one or both of these agents. Combination therapy by co-administration of individual sst_2/sst_5 or D_2-targeting compounds or by novel dopastatin chimeras, appears to be a feasible approach, which is supported by different *in vitro* studies. The decrease in circulating cortisol levels may induce a secondary beneficial effect of upregulation of sst_2 expression in these adenomas, which further enhances pharmacotherapeutical options in these patients. Preliminary data from an ongoing clinical trial, which uses a stepwise approach of pasireotide (sst_2/sst_5), cabergoline (D_2) and low-dose ketoconazole, are supportive for the efficacy of combining these partially independent medical therapies in the treatment of human Cushing's disease.

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Chapter 9

Summary

Samenvatting

SUMMARY

Cushing's disease (CD) is a rare endocrinological disorder due to an ACTH-producing adenoma in the pituitary gland. Although the clinical presentation is highly variable in terms of type and severity of symptoms, the disease invariably has grave consequences for the physical and emotional well being of patients. Despite the fact that neurosurgical removal of the adenoma is the first choice of treatment, only 70% of patients with CD can obtain long-term cure by surgery alone. Adjuvant radiotherapy and bilateral adrenalectomy are both effective secondary treatments, but have considerable disadvantages and side effects. Therefore, an effective and safe medical treatment for patients with persistent or recurrent CD could provide an important addition to the current management of these patients. At present, the role of medical therapy for CD is limited, mainly due to a lack of treatment efficacy or unfavourable safety profiles, but new approaches have been sought. Dopamine and somatostatin receptors have been identified as novel targets for a medical therapy of CD, but data on their clinical applicability are still limited. The primary aim of the present thesis was to further investigate the future potential of medical therapies that target dopamine and/or somatostatin receptor subtypes in patients with CD.

Chapter 1 provides an overview of the pathophysiology and current treatment of Cushing's disease. It also describes the current knowledge on somatostatin and dopamine receptors in the human neuro-endocrine system and especially in Cushing's disease.

In **Chapter 2** we investigate the possibility of using dogs with Cushing's disease (canine Cushing) as a model to study human CD. In contrast to humans, CD is a very common disorder in dogs, but only a few veterinary centers worldwide can perform transsphenoidal surgery for this indication. The abundance of pure corticotroph adenoma tissue that is surgically removed during this type of surgery could be of great benefit for the study of human CD. It could help in determining the efficacy of various novel compounds that are considered for use in human CD. In this study we investigated the functional expression of the somatostatin (sst_2 and sst_5) and dopamine (D_2) receptor subtypes in 13 canine corticotroph adenomas. We found that canine and human corticotroph adenomas both express these receptor subtypes, but that distinct differences do exist, so that direct comparisons between humans and canine CD can not be made. In spite of these differences, however, several future research questions in human CD could very well be addressed by the use of canine corticotroph adenoma tissue.

Chapter 3 describes the studies we have performed to investigate the role of glucocorticoids on expression levels of sst_2, sst_5 and D_2 in human neuro-endocrine tumor cell lines.

We found that glucocorticoids strongly down-regulate sst_2, while sst_5 and especially D_2 are relatively resistant to this type of down-regulation. These results may explain the observed sst and D_2 expression levels in corticotroph adenomas of patients with CD who suffer from sustained hypercortisolism (e.g. low sst_2, high sst_5 and D_2) and are also in line with earlier clinical studies that have shown marked inefficacy of sst_2-preferring compounds such as octreotide in patients with CD, while sst_5 and D_2 targeting drugs are generally more effective. We also observed that this glucocorticoid-mediated down-regulation of sst_2 is a reversible phenomenon *in vitro*. This means that initial glucocorticoid lowering therapy in patients with CD may increase the expression of sst_2 receptors within the adenoma and consequently increase the pharmacological options in these patients.

Although some studies had already investigated the individual expression patterns of SS and DA receptor subtypes in patients with CD, these data were derived from relatively small patient series and co-expression data were not available. In **chapter 4** we have investigated the expression of both receptor types in a relatively large number of human corticotroph adenomas (n=30). We found that more than 80% of these adenomas express sst_5 and/or D_2 at significant levels, which makes them potential candidates for sst_5/D_2-directed medical therapy. An important finding in this study was that large, invasively growing macroadenomas appear to be less suitable for this type of therapy, due to a general absence of sst_5/D_2 expression in these specific adenomas. Apart from adenoma stage, we were not able to identify pre-operative clinical correlates, which could predict sst_5 or D_2 status in patients diagnosed with de novo or recurrent CD.

In **chapter 5** we show some early results from an ongoing multicenter clinical trial, which investigates the efficacy of combined sst_2/sst_5 and/or D_2 targeted medical therapy in human patients with CD. This study uses a stepwise medical approach with the use of the novel multiligand SS receptor analogue pasireotide, the D_2 agonist cabergoline and/or low-dose ketoconazole in de novo or recurrent CD patients. In this chapter we describe four patients who participated in this study and who showed a large variation in treatment responses.

Apart from pituitary-derived hypercortisolism (CD), patients with ectopic ACTH-producing Cushing's syndrome (EAS) could benefit from SS or DA receptor targeted therapies as well. In **chapter 6**, we describe our clinical and *in vitro* findings in a patient who presented in our clinic with EAS due to an ACTH-secreting bronchial carcinoid tumor. We found that six months of glucocorticoid receptor antagonizing therapy by mifepristone, caused a reappearance of sst_2 receptors in this tumour, which was previously invisible on ^{111}In-Pentreotide scintigraphy (Octreoscan). We hypothesize that the subset of EAS

tumors, which still display partial glucocorticoid responsiveness in vivo, could benefit from initial glucocorticoid lowering or antagonizing therapy to improve diagnostic accuracy (^{111}In-Pentreotide scintigraphy) and to enhance potential treatment response to sst_2 targeting therapies (Octreotide).

The interest in combined targeting of sst and DA receptors in the treatment of human neuro-endocrine tumours has led to the development of novel dopamine-somatostatin chimeric compounds, which combine high binding affinities for both receptors. In **chapter 7** we have investigated the *in vitro* efficacy of one these chimeric compounds, BIM-23A760, which has displayed superagonistic properties in earlier preclinical studies. We found that in comparison to its reported binding affinities, this compound was a remarkably potent activator of D_2, while its efficacy via sst_2 was lower than expected. Combined targeting of both receptors *in vitro* did not result in enhanced potency, suggesting that at least some of the superagonistic properties of BIM-23A760 are likely to be due to increased activation of individual receptors.

In **chapter 8**, the general discussion, the results of these studies are further discussed. Special emphasis is given to those issues, which will ultimately determine the clinical applicability of future medical therapies that target sst_2, sst_5 and D_2 receptors in patients with human CD.

SAMENVATTING

De ziekte van Cushing is een zeldzame endocrinologische aandoening, die veroorzaakt wordt door een ACTH-producerend adenoom in de hypofyse. Hoewel de klinische presentatie en de ernst van de symptomen sterk per patiënt kan variëren, heeft de ziekte doorgaans ernstige consequenties voor de lichamelijke en geestelijke gezondheid voor patiënten die lijden aan deze aandoening. Hoewel neurochirurgische verwijdering van het adenoom via de transsphenoidale route de behandeling van eerste keus is, kan slechts bij 70% van de patienten blijvende genezing bereikt worden door middel van deze operatie alleen. Het geven van aanvullende radiotherapie en/of het chirurgisch verwijderen van de bijnieren, zijn beide effectieve secundaire behandelingsvormen, maar worden tevens gekenmerkt door aanzienlijke nadelen en bijwerkingen. Om die reden zou de ontwikkeling van een effectieve en veilige medische therapie een belangrijke aanvulling betekenen in het huidige arsenaal van behandelingsmogelijkheden, met name in de subgroep van Cushing patiënten, bij wie de ziekte persisteert of recidiveert na een eerste operatie. Op dit moment is de rol van medische behandeling voor de ziekte van Cushing nog zeer beperkt, met name door een gebrek aan behandelingseffect en ongunstige bijwerkingsprofielen, maar nieuwe invalshoeken zijn in ontwikkeling. Inmiddels kunnen dopamine en somatostatine receptoren gezien worden als mogelijke nieuwe aangrijpingspunten voor een medische behandeling voor de ziekte van Cushing, maar data over hun klinische toepasbaarheid is vooralsnog beperkt. Het hoofddoel van de huidige dissertatie was om verder te onderzoeken wat het toekomstig potentieel is van medische therapieën die gericht zijn op dopamine en/of somatostatine receptor subtypen bij patiënten met de ziekte van Cushing.

Hoofdstuk 1 geeft een overzicht van de pathofysiologie en de huidige behandeling van de ziekte van Cushing. Het beschrijft ook de reeds aanwezige kennis over de rol van somatostatine en dopamine receptoren in het humane neuro-endocriene systeem en met name bij de ziekte van Cushing.

In **Hoofdstuk 2** onderzoeken we of honden die aan de ziekte van Cushing lijden ("canine Cushing"), model kunnen staan voor humane patiënten met de ziekte van Cushing. In tegenstelling tot bij mensen is de ziekte van Cushing een frequent voorkomende aandoening bij honden. In een zeer beperkt aantal klinieken ter wereld, waaronder de Universiteit van Utrecht, kunnen honden door middel van transsphenoidale chirurgie behandeld worden voor deze aandoening. Het pure corticotrofe adenoomweefsel dat tijdens een dergelijke operatie in aanzienlijke hoeveelheden verwijderd wordt, zou van grote waarde kunnen zijn voor het onderzoek naar de humane ziekte van Cushing. Zo zou het verwijderde weefsel *in vitro* gebruikt kunnen worden om de effectiviteit te bepalen van diverse nieuwe medicamenten, die overwogen worden voor gebruik bij mensen

met de ziekte van Cushing. In deze studie hebben we de functionele expressie onderzocht van somatostatine receptor subtypen 2 en 5 (sst_2 en sst_5) en dopamine receptor subtype 2 (D_2) in 13 gevallen van canine (=hond) Cushing. We vonden dat zowel canine als humane corticotrofe adenomen deze receptor subtypen tot expressie brengen, maar dat er specifieke verschillen bestaan, waardoor directe vergelijkingen tussen de humane en canine vorm van de ziekte van Cushing niet gemaakt kunnen worden. Ondanks deze distincte verschillen, zal het corticotrofe adenoom weefsel van honden zeer goed gebruikt kunnen worden voor het beantwoorden van diverse andere onderzoeksvragen in het onderzoek naar de humane vorm van de ziekte van Cushing.

Hoofdstuk 3 beschrijft de studies die we verricht hebben naar de effecten van glucocorticoiden (GC's) op de expressie van sst_2, sst_5 en D_2 in drie humane neuro-endocriene cellijnen. We hebben gevonden dat GC's in sterke mate de expressie van sst_2 down-reguleren, terwijl sst_5 en met name D_2 veel resistenter zijn tegen dit type down-regulatie. Deze resultaten zouden de sst en D_2 expressie niveaus kunnen verklaren (laag sst_2, hoog sst_5 en D_2), die in de corticotrofe adenomen gevonden worden van patienten met de ziekte van Cushing, aangezien deze blootgesteld worden aan langdurig hypercortisolisme. Daarnaast zijn deze resultaten in overeenstemming met eerdere klinische studies, die een opmerkelijk lage effectiviteit hebben aangetoond van sst_2-gerichte medicamenten zoals Octreotide, terwijl sst_5 en D_2-gerichte therapieën doorgaans effectiever zijn. Verder vonden we dat deze glucocorticoid-gemedieerde down-regulatie een reversibel proces *in vitro* is. Dit betekent dat initiële glucocorticoid-verlagende therapie in patiënten met de ziekte van Cushing de expressie niveaus van sst_2 in het adenoom zouden kunnen verhogen, waarmee de pharmacotherapeutische opties in deze patiënten aanzienlijk vergroot worden.

Hoewel enkele andere studies reeds de individuele expressie patronen van somatostatine en dopamine receptor subtypen in patiënten met de ziekte van Cushing hadden onderzocht, waren deze data afkomstig van relatief kleine series patiënten en waren co-expressie data niet beschikbaar. In **Hoofdstuk 4** hebben we de simultane expressie van beide typen receptoren onderzocht in een relatief grote serie humane corticotrofe adenomen (n=30). Hieruit bleek dat meer dan 80% van deze adenomen sst_5 en/of D_2 receptoren significant tot expressie brengt, waarmee dit potentiële kandidaten zijn voor sst_5/D_2 gerichte medische therapie. Een belangrijke waarneming in deze studie was dat grote, invasief groeiende macroadenomen minder geschikt lijken te zijn voor dergelijke therapie, als gevolg van een algehele afwezigheid van sst_5/D_2 receptor expressie in deze specifieke adenomen. Afgezien van het stadium van het adenoom, konden we geen andere pre-operatieve klinische variabelen identificeren, die voorspellend zijn voor de sst_5 of D_2 status in patiënten die gediagnosticeerd worden met de novo of recidief ziekte van Cushing.

In **Hoofdstuk 5** hebben we een aantal eerste resultaten laten zien van een lopende multicenter klinische studie, die de effectiviteit onderzoekt van gecombineerde sst_2/sst_5 en/of D_2 gerichte medische therapie in humane patiënten met de ziekte van Cushing. Deze studie gebruikt een stapsgewijze medische benadering met het nieuwe multiligand somatostatine receptor analogon pasireotide, de D_2 agonist cabergoline en lage dosis ketoconazol in patiënten met de novo of recidief ziekte van Cushing. In dit hoofdstuk staan vier patiënten beschreven die deel hebben genomen aan deze studie en die een grote variatie in respons op deze therapie hebben laten zien.

Naast de hypofyse-afhankelijke vorm van hypercortisolisme (de ziekte van Cushing), zouden patiënten met het ectopisch ACTH-producerend syndroom (EAS, ook wel Ectopisch Cushing syndroom genoemd) kunnen profiteren van somatostatine of dopamine receptor gerichte therapieën. In **Hoofstuk 6** hebben we onze klinische en *in vitro* bevindingen beschreven in een patiënt die zich in onze kliniek presenteerde met EAS, welke veroorzaakt werd door een ACTH-secernerend bronchus carcinoid. Wij zagen dat 6 maanden behandeling met glucocorticoid receptor antagonerende therapie in de vorm van mifepristone, leidde tot een terugkeer van sst_2 receptoren in deze tumor, die voordien onzichtbaar was op ^{111}In-Pentreotide scintigraphie (OctreoScan). Wij denken dat de subgroep van EAS tumoren, die *in vivo* nog enige mate van glucocorticoid responsiviteit toont, zou kunnen profiteren van initiële glucocorticoid verlagende of antagonerende therapie door enerzijds het vergroten van de diagnostische gevoeligheid van ^{111}In-Pentreotide scintigraphie (OctreoScan) en anderzijds het versterken van de mogelijke respons op sst_2 gerichte therapieën (Octreotide).

De interesse in het gelijktijdig activeren van somatostatine en dopamine receptoren in de behandeling van humane neuro-endocriene tumoren heeft geleid tot de ontwikkeling van nieuwe dopamine-somatostatine chimere moleculen, die een hoge affiniteit voor beide receptoren bevatten. In **Hoofdstuk 7** hebben we de *in vitro* effectiviteit van één van deze chimere moleculen onderzocht, BIM-23A760, welke in eerdere preklinische studies superagonistische eigenschappen heeft laten zien. We hebben gevonden dat in vergelijking met de opgegeven bindingsaffiniteiten van dit molecuul, BIM-23A760 een opmerkelijk krachtige activator van de D_2 receptor is, terwijl dat voor sst_2 juist minder is dan verwacht. De simultane activatie van beide receptoren *in vitro* resulteerde niet in toegenomen effectiviteit, hetgeen suggereert dat in ieder geval een deel van de superagonistische eigenschappen van BIM-23A760 verklaard kunnen worden door een verhoogde activatie van individuele receptoren.

In **Hoofdstuk 8**, de algemene discussie, worden de resultaten van bovengenoemde studies verder besproken. Hierbij wordt de nadruk gelegd op die aspecten, die uiteindelijk

de klinische toepasbaarheid zullen gaan bepalen van toekomstige medische therapieën die gericht zijn op sst_2, sst_5 en D_2 receptor activatie in patiënten met de ziekte van Cushing.

Appendix

Cushing's disease in dogs and humans

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ABSTRACT

Background*:* Cushing's disease (CD) is a common endocrinological disorder in dogs with an estimated incidence of 1-2 cases/l000 dogs/year. This is in contrast to humans in whom CD is rare. The clinical presentation of CD, however, is highly similar between dogs and humans, with characteristic signs, such as abdominal obesity, weight gain, fatigue, muscle atrophy and skin changes. Canine CD may therefore serve as an animal model for human CD, especially since therapeutic canine hypophysectomy can generate substantial amounts of primary corticotroph adenoma tissue for in vitro research purposes. In a recent study, we found that dopamine (DA) D_2 and somatostatin (SS) receptor subtypes are well expressed in canine corticotroph adenomas, but there are some distinct differences compared with the expression profile observed in human CD. These differences need to be considered when using canine CD as a model to evaluate the efficacy of novel DA/SS compounds for potential use in human CD. **Case Report:** This case involves an 8-year-old female dog that developed signs of exercise intolerance, muscle weakness and polyuria/polydipsia due to an adrenocorticotropic hormone-secreting pituitary adenoma. The dog underwent curative transsphenoidal hypophysectomy and has remained in complete remission in the 3.5 years since surgery.

INTRODUCTION

Cushing's disease (CD) is a severe endocrinological disorder due to an adrenocorticotropic hormone (ACTH)-secreting adenoma in the pituitary gland. Patients with CD suffer from the effects of sustained hypercortisolism, such as truncal obesity, diabetes mellitus, osteoporosis, hypertension and psychiatric disturbances [1]. In humans, CD is a rare disorder with an estimated incidence of 1.2 -2.4 new cases/million/year [2, 3].

CD is also known to occur in several animal species, including horses and dogs. In fact, canine CD is one of the most common endocrinological disorders encountered in general veterinary practice. Exact incidence figures are lacking because there is no formal registration system, but some epidemiological studies have estimated its incidence to be 1-2 cases/1000 dogs/year [4]. Standard treatment of canine CD consists of medical therapy with drugs such as mitotane or trilostane. Another option is to perform transsphenoidal pituitary surgery. At present this option is only routinely available at a limited numbers of centers worldwide. We now present such a case.

CASE PRESENTATION

The canine patient is an 8-year-old female Belgian shepherd dog. The owner had witnessed a gradual decline in the dog's condition over the course of the previous year, especially in terms of decreased physical activity. In addition, the owner had noticed a remarkable change in drinking pattern, which was estimated to be around 10 L/day (normal water intake corrected for breed, age and body weight: 2-4 L daily). On physical examination, the dog had mild abdominal obesity and pronounced atrophy of the leg and lumbar muscles. There was no alopecia or skin atrophy. Furthermore, the dog was panting while at rest, which is abnormal for dogs not participating in any physical activity.

Based on these clinical signs, a preliminary diagnosis of hypercortisolism (Cushing's syndrome) was considered and further laboratory investigation was undertaken. Morning urine samples collected on 2 consecutive days showed elevated urinary corticoid:creatinine ratios (UCCRs) of 69 and 72 $\times 10^{-6}$, respectively (normal range: < 10 $\times 10^{-6}$). To distinguish between a pituitary versus adrenal cause of the hypercortisolism, high doses of dexamethasone (DEX) were administered after collection of the second urine sample (three doses of 0.1 mg/kg at 8-h intervals). The next morning, a third urine sample showed DEX-induced suppression of the UCCR to 6.7 $\times$ l0^{-6} (> 50% suppression), which strongly suggested a functional corticotroph pituitary adenoma. Random blood

samples (average of two independent measurements drawn 15 min apart) confirmed the diagnosis of pituitary-dependent hypercortisolism. The mean plasma concentration of cortisol was 141 nmol/l (normal range: 11–136 nmol/l), ACTH was 17.9 pmol/l (normal range: 1.1–18.7 pmol/l) and α-melanocyte-stimulating hormone (α-MSH) was 23.4 pmol/l (normal range: < 22 pmol/l). As a final diagnostic step, a computed tomography scan of the pituitary fossa was performed under general anaesthesia. This showed an adenoma within an enlarged pituitary of 7.3 mm (height) × 6.9 mm (width) × 8.0 mm (length) (see figure 1).

The dog underwent transsphenoidal hypophysectomy with complete removal of the pituitary gland including the adenoma. The postoperative phase was unremarkable with rapid clinical improvement and discharge from the hospital on the second day after surgery. Postoperative replacement therapy consisted of desmopressin for 4 weeks and lifelong treatment with cortisone acetate and levothyroxine. Histological examination of the resected pituitary tissue revealed a basophilic adenoma, which stained positive for ACTH and α-MSH on immunohistochemistry. Two weeks after surgery, UCCRs were measured after temporary withdrawal (24 h) of the cortisone acetate. These values were very low (0.8-1.0 × 10^{-6}), which strongly suggested complete remission of the hypercor-

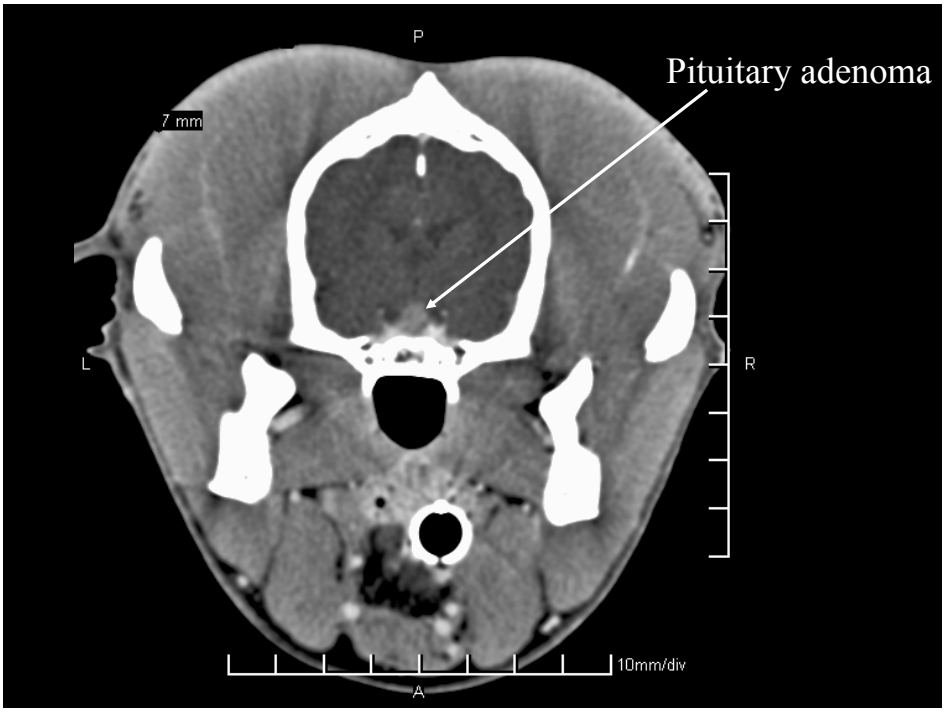

Figure 1

tisolism. In the months thereafter, a gradual return of full physical activity and muscular strength occurred and the drinking pattern also normalized. At present, 42 months after surgery, the dog is in very good condition and UCCR values during follow-up have remained within the normal range.

DISCUSSION

As illustrated by this case, many similarities exist between the clinical presentation of CD in dogs and humans (see table 1). Fatigue, weight gain, truncal obesity and muscle atrophy are cardinal signs in both species [1, 5]. In both humans and dogs, approximately 70% of CD cases are due to a pituitary adenoma, while the remaining cases are due to adrenal or ectopic causes. Subtle differences are present as well. Human CD patients often develop osteoporosis and hirsutism while dogs do not, and the main clinical sign of canine CD is polyuria/polydipsia, which is not observed in humans. The major difference, however, between dogs and humans is the large difference in incidence rates: 1-2 cases/1000 dogs/year compared with 1.2-2.4 cases/million humans/year. The causes of the remarkable susceptibility of dogs to develop this endocrine disorder are still unknown.

The high natural incidence of canine CD and its many clinical similarities to human CD raises the question whether canine CD could serve as a spontaneous animal model for human CD. This question is particularly important because at present there is no accepted animal model for this deleterious disease and there are no human ACTH-producing cell lines to facilitate in vitro research into the pathogenetic mechanisms of CD. Moreover,

Table 1 Characteristics of human and canine Cushing's disease

Clinical sign	Human	Canine
Fatigue	+	+
Weight gain	+	+
Truncal obesity	+	+
Muscle atrophy	+	+
Osteoporosis	+	-
Lordosis	-	+
Hypertension	+	+
Polyuria / polydipsia	-	+
Thinning of skin	+	+
Easy bruising	+	-
Hirsutism	+	-
Alopecia	+/-	+

due to the low incidence of the disease, human primary corticotroph tissue obtained at transsphenoidal surgery is difficult to obtain, which can further slow advancements in research. From this perspective, the establishment of transsphenoidal hypophysectomy as an accepted treatment for canine CD may have opened up a new source of valuable primary corticotroph tissue, which could overcome some of the these obstacles [6, 7]. Van Wijk et al. have shown previously that canine corticotroph adenoma cells cultured in vitro retain important biological properties, such as the production of ACTH, and a variable responsiveness to both corticotropin-releasing hormone and glucocorticoids, just like human corticotroph adenoma cells in vitro [8]. Canine corticotroph adenomas also have been used to study fundamental aspects of the impaired feedback regulation of the glucocorticoid receptor in CD [9].

To fully evaluate the feasibility of using canine CD as a direct model for human CD, it is necessary to understand the molecular make-up of these canine corticotroph adenomas and to compare this with what is known about human corticotroph adenomas. To this end, we recently performed a study in which we investigated the expression of dopamine (DA) and somatostatin (SS) receptor subtypes in canine corticotroph adenomas [10]. We specifically investigated these receptor subtypes because compounds that target them have been shown to reduce ACTH and cortisol levels in subsets of human CD patients [11, 12]. Our study showed that DA and SS receptor subtypes were functionally expressed in these canine adenomas, but with some distinct differences compared with human adenomas. In particular, SS receptor subtype 2 (sst_2) was highly expressed in canine adenomas, whereas its expression in human adenomas is very low, probably due to glucocorticoid-induced downregulation [13]. In the canine adenomas, D_2 was also moderately well expressed, while sst_5 expression was remarkably low, which is different from human adenomas [14, 15]. The expression pattern in canines was confirmed by quantitative polymerase chain reaction, immunohistochemistry and functional studies measuring ACTH release under the influence of specific DA/SS analogues.

IMPLICATIONS

These findings have implications for the use of canine corticotroph adenomas as a direct model for human CD. With respect to SS and DA receptors, direct extrapolations cannot be made regarding the efficacy of novel SS/DA compounds for future use in human CD patients. Based on our findings, it is likely that canine corticotroph adenomas will respond differently in vivo to specific SS/DA analogues compared with what we have observed previously for humans. However, canine CD could provide a unique opportunity to directly test the in vitro and in vivo ACTH-inhibiting capacities of novel compounds

such as the DA-SS chimeric molecules, for which data from primary corticotroph cell systems are still limited. The canine corticotroph model also would enable more thorough investigation of secondary or auxiliary factors that determine the level of ACTH inhibition induced by treatment with SS/DA-targeting compounds in primary corticotroph cells. Furthermore, it would be interesting to characterize canine corticotroph adenomas for the functional expression of other receptors known to regulate ACTH release, such as retinoic acid receptor and peroxisome proliferator-activated receptor-γ [16]. In this way, we might gain a better understanding of the full spectrum of molecular differences and similarities between canine and human corticotroph adenomas and consequently increase our knowledge of the possibilities and limitations of using canine CD as a model for human CD.

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1998-1999	Research elective tumorangiogenesis (8 months), Georgetown University, Washington DC, U.S.A. (Supervisor: Professor Wellstein): Nijbakker-Morra student research prize.
2000	Summer Course Epidemiology (1 month), Boston, U.S.A.
2001	Clinical clerkships at Triemli Stadtspital Zurich, Switzerland and Universita degli studi di Parma, Italy (5 months)
2004	Medical degree (cum laude)
2004-2005	Resident (ANIOS) in pediatrics (MCRZ, Rotterdam) and internal medicine (GHZ, Gouda)
2005	USMLE certification (Step 1: 227; Step 2: 230; Step 2 CS passed)
2005-present	Research fellow (PhD program) at Department of Endocrinology, Erasmus MC, Rotterdam (Supervisors: Professor Lamberts and Dr. Hofland)
2009-present	Pediatric residency training (AIOS) at Rijnstate Hospital Arnhem and UMC St. Radboud Nijmegen