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Effect of SOM230 (Pasireotide) on Corticotropic Cells: Action in Dogs with Cushing's Disease

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Key Words
SOM230 · Cushing's disease · Corticotropes · Pituitary adenoma

Abstract
SOM230 (pasireotide) is a multiligand somatostatin (SRIF) analog able to bind to somatostatin receptor (SSTR) subtypes 1, 2, 3 and 5, and trigger antisecretory and antiproliferative signaling cascades. Canines have become in vivo models to test the pharmacological treatment of corticotropinomas because they frequently develop Cushing's disease in a spontaneous manner, due to adrenocorticotropic hormone (ACTH)-producing pituitary adenomas. Different levels of expression of SSTR2 and SSTR5 have been shown in both mouse AtT20 cells and canine tumoral corticotropinoma cells. The objective of this study was to evaluate whether SOM230 controls both tumor cell growth and hormone synthesis, therefore controlling the disease. SOM230 was tested in dogs suffering from Cushing's disease (10 animals were treated continuously during 6 months, and another 10 were treated with 3 cycles consisting of 2 months of treatment followed by a 2-month rest period). A significant decrease in ACTH, urinary cortisol creatinine ratio, adenoma size (magnetic nuclear resonance) and improvement of clinical signs were obtained, without side effects. AtT20 cells treated with SOM230 suppressed pro-opiomelanocortin (POMC) promoter activity through SSTR2, via the G_i α-subunit, and reduced Nur77/Nurr1 transcriptional activity. We conclude that SOM230, in addition to its well-described antisecretory effects, inhibits, as shown in AtT20 cells, ACTH synthesis at the POMC transcriptional level, an effect mediated mainly through SSTR2, and limits tumor growth. The controlled Cushing's disease in the dogs that received the treatment indicates that SOM230 has a potential therapeutic use in humans suffering from Cushing's disease.

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Introduction

Pharmacological therapy for Cushing's syndrome caused by adrenocorticotropic hormone (ACTH)-producing pituitary adenomas (corticotropinomas) has been

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an object of intense research in recent years. The use of drugs would be useful as a complement to surgery or radiotherapy in order to avoid new tumor formation or side effects [1–3], and essential when surgery or radiotherapy are not possible. Corticotropinoma pathogenesis is marked by alterations in factors regulating cell cycle progression, pro-opiomelanocortin (POMC) transcription and ACTH synthesis [4–9] that constitute putative drug targets [reviewed in 10]. Indeed, targeting POMC transcription and corticotropinoma cell viability with retinoic acid was able to reverse Cushing's disease in experimental animal models [11, 12]. In addition, corticotropinoma cells were shown to express dopamine D_2 (D2DR) [13] and somatostatin receptors (SSTR1, 2, 3 and 5) [14–16], making them candidates for treatment with dopamine agonists, such as cabergoline and somatostatin analogs, e.g. octreotide and lanreotide. Treatment with cabergoline was shown to be effective in animal models and patients with Cushing's disease [17–19].

In contrast, the somatostatin analog octreotide was not effective in controlling pituitary-dependent Cushing's disease [20, 21]. In vitro studies demonstrated that SSTR5 is the primary receptor suppressing ACTH secretion [15, 16, 22, 23]. Octreotide has a high affinity for SSTR2 and less for SSTR5, explaining in part the lack of a potent suppressive effect on ACTH synthesis in human corticotropinomas [21]. In addition, human corticotropinomas express SSTR2 at moderate levels, with SSTR5 being the prominent receptor [14]. Interestingly, dexamethasone was shown to decrease SSTR2, but not SSTR5, transcription [15, 24], indicating that the high circulating glucocorticoid levels in patients with Cushing's disease may be responsible for the relatively low SSTR2 expression in corticotropinomas. Altogether, these observations indicate that somatostatin analogs, which are able to target receptors other than SSTR2, could be more potent in suppressing ACTH synthesis. Multireceptor-targeted somatostatin analogs, such as SOM230 (pasireotide), which has high affinity for SSTR2, 3 and 5, and less for SSTR1 [25], would be more potent in controlling aberrant corticotropinoma growth and function.

Recently, canine corticotropinomas were shown to express dopaminergic D_2 receptors and different subtypes of SSTRs, with SSTR2 being the predominant subtype and SSTR5 the least expressed. Expression of mRNA for SSTR2 and 5 has been studied in 13 dogs with Cushing's disease and showed primarily an overall stronger expression of SSTR2 versus SSTR5 mRNA. To which extent these differences translate also into similar differences in expression of the respective functional receptor subtype can currently not be assessed, due to the lack of dog-specific antibodies against SSTR5 receptor subtype [24]. Contrary to what was reported in vitro with AtT20 cells, in canine corticotropic cells, SSTR2 expression is increased in the presence of dexamethasone. As in human corticotropic cells treated with octreotide, a study in healthy dogs and those with insulinoma proved that this drug does not affect cortisol and ACTH concentration in the blood [26]. Since dogs are animals that frequently suffer of Cushing's syndrome, being of pituitary origin in 80% of the cases [27, 28], they are considered a model for the study of different aspects of this disease [12]. Although quantitative differences in the expression of SSTR subtypes may exist in corticotropinomas of humans and dogs, it is conceivable that a multireceptor ligand like SOM230 might have a stronger effect in dogs with Cushing's disease than currently available somatostatin analogs. Our objective is to study the action of the SSTR multiligand, SOM230, in corticotropinomas and test its action in controlling Cushing's disease in dogs that spontaneously developed this condition.

Materials and Methods

Reagents

SOM230 (Novartis Pharma AG, Basel, Switzerland) was dissolved in 0.01 M acetic acid to give 10^{-3} mol/l stock solutions, which were stored at –20°C. A new aliquot was thawed and used for each cell culture experiment.

Population under Study

A total of 20 dogs, of different breeds and crosses, diagnosed with ACTH-dependent Cushing's disease caused by corticotropinoma, were used for this study. The average age was of 8.5 ± 2.2 (range 5–13) years, 7 males and 13 females (3 neutered). Disease diagnosis was determined with the following criteria, as previously described [12]: (1) Presence of at least 5 clinical signs characteristic of Cushing's syndrome, 3 of which were of objective value (polydipsia-polyuria, absence of estrous cycle in females for more than 1 year, weight gain) and the last 2 signs of subjective value (i.e. according to the veterinarian's and owner's impression: polyphagia, abdomen prominence, skin and hair alterations). (2) Urinary cortisol creatinine ratio (UCCR) values >70, according to the methodology implemented by Unidad de Endocrinología, Hospital Escuela de Medicina Veterinaria, Facultad de Ciencias Veterinarias-UBA. Urine samples were collected as follows: urine was collected by the dog owners, during the moment the dog was urinating (the urine directly fell into the container). After collecting the urine, the owners took 1 ml of it and put it into another container where all of the daily urine was being collected, and kept it refrigerated. This way, there were equal volumes of each micturition. Once the total urine of the day was collected, it was frozen at –20°C until sent to the laboratory for analysis. (3) Plasma ACTH measurements, considering the reference value 65 pg/

ml with normal UCCR as acceptable, while pathological values were considered to be inappropriately high values of ACTH in relation to high UCCR. (4) Presence of pituitary adenoma, determined by nuclear magnetic resonance imaging (NMRI). Dogs that presented clinical evidence of other diseases at the time of the diagnosis, whether they were of organic (cardiopathy, neuropathy, diabetes mellitus or glycemias >125 mg/dl, epilepsy, etc.) or infectious type, systemic or local, were excluded from this study.

Given the possibility that a decreased response to SOM230 could occur because of desensitization or a drug resistance developed by the tumor cells, two comparative protocols were established: one consisting of continuous application and another alternating of rest periods in between treatment periods. Accordingly, the dogs were distributed in two treatment groups with SOM230, with the following experimental design:

Treatment group 1 (TG1): The first 10 dogs diagnosed, 9 female (6 not neutered) and 1 non-neutered male. They were administered one dosis of 0.03 mg/kg [29] SOM230, subcutaneously, every 12 h, continuously during 6 months. Clinical and biochemical variables were evaluated at different times: before treatment (B) and at 3 and 6 months (3 m, 6 m) of treatment. NMRI studies at B and 6 m were performed.

Treatment group 2 (TG2): The next 10 dogs diagnosed, 4 non-neutered females and 6 non-neutered males. They were administered the same dosis of SOM230 but with therapeutic cycles which alternated 2 months of continuous treatment with a 2-month rest period. A total of 3 cycles of SOM230 and 2 cycles of drug arrest between the first and second cycles and between the second and third cycles of treatment were performed, meaning a total duration of 10 months' treatment. Clinical and biochemical evaluations were performed on B and at the end of each cycle (2, 6 and 10 m under SOM230 treatment and 4 m and 8 m of the rest cycles). The NMRI was performed at times B and 10 m.

Biochemical and Endocrine Studies

Plasma ACTH, α-MSH and UCCR measurements were carried out at the mentioned times. ACTH (ImmuChem Double Antibody hACTH ^{125}I RIA kit; MP Biomedicals, Orangeburg, N.Y., USA) and α-MSH (Euro-Diagnostica, Malmö, Sweden) were measured by the RIA method. The intra-assay variation coefficients for the ACTH kit were 6.0 and 4.1% for mean values of 40.9 and 139 pg/ml, respectively, while the inter-assay variation coefficients were 10.7 and 4.0% for mean values of 6.3 and 107.8 pg/ml, respectively. Samples were collected in EDTA and plasma was stored in Trasylol at –80°C. According to the background information of the manufacturer, this assay may be used in other species like dogs to detect ACTH. There is no cross-reactivity with other POMC-processed hormones. Dilutions of the samples were on the standard curve, and were above the lowest limit of detection (8 pg/ml). For the α-MSH kit, the intra-assay variation coefficients were 11.8, 4.7 and 2.9% for 16.2, 33.6 and 77.7 pmol/l, respectively, while the inter-assay variation coefficients were 13.0, 8.4 and 4.0% for 16.5, 37.8 and 79.6 pmol/l, respectively. Blood extraction was performed in a refrigerated plastic tube containing EDTA and Trasylol, in order to stabilize the ACTH and α-MSH molecules. The samples were centrifuged immediately and the plasmas were stored at –80°C until their processing.

UCCR was determined by solid-phase radioimmunoassay (DPC Corp., San Diego, Calif., USA), with intra- and inter-assay variations of 8 and 5%, respectively. Urinary aliquot recollection was performed as described in 'Population under Study'.

Diagnostic Imaging

Magnetic resonance images (at the times indicated according to the treatment group) were obtained as previously described [12, 28], using a General Electric 1 Tesla closed resonance machine, in sagittal, coronal and axial sections, using gadolinium as a contrast agent.

Tumor size evaluation was performed with the middle line of the sagittal section, measuring the tumor from the base up to its highest point. Two NMRIs were performed on the dogs: at the time of diagnosis and at the end of treatment. These were performed under total anesthesia, with the indicated 2-mm sections. For adenoma size measurement, the height of the adenoma was taken (as described) and evaluation of whether it surpasses the sella turcica or not was performed (intra- or extrasellar, therefore making it independent of the different sizes the dogs may have, even though the study was performed on dogs of similar sizes). The intensity of the paramagnetic signal (hyper- or isointense compared to the rest of the encephalic tissue) served as an indicator of adenoma activity. The adenoma is distinguished, as what appears in the image with greater paramagnetic signal intensity than the rest of the encephalic parenchyma and the pituitary. Adenoma classification is as follows: tumors which were intrasellar (<5 mm) were considered microadenomas, while those which were extrasellar (>5 mm) were considered macroadenomas [19, 28].

Assessment of Frequent Clinical Signs in Dogs with Cushing's Disease

(1) Signs of objective assessment: weight, return of estrous cycles in females, fluid uptake (normal up to 100 ml/kg/day [30]) and micturition (signs of polydipsia and polyuria).

(2) Signs of subjective assessment: solid food uptake (polyphagia or normal), skin aspect (width, elasticity) and hair (structure alteration, unctuosity, slight to severe loss), and abdomen aspect (from slight to severe prominence or normal). With the exception of weight and return of a normal estrous cycle, the rest of the signs were evaluated using the following scale of 1–5: 1 = very good, 2 = good, 3 = stable, became better or worst with respect to the previous evaluation, 4 = bad, and 5 = very bad. This evaluation was performed as follows: the dogs of each treatment group were evaluated by two groups of three different veterinarian interns, on different days (each one registering their clinical evaluation without the other colleagues taking knowledge of it), and without knowing to which treatment group the dog under evaluation pertained to (i.e. TG1 or TG2), or the length of treatment up until the moment of their evaluation. The interns belonging to each observation group were exchanged between the two groups every month, in order to avoid bias when evaluation of the same dog took place the following month. If the evaluations differed among the three veterinarians (whether all three were different among each other or two concorded and one was different), the average was calculated. With these procedures, bias was avoided.

Diabetes was assessed clinically with the following signs: glycemia >170 mg/dl, polydipsia-polyuria, polyphagy and rapid weight loss. In the event of diabetes, insulin detemir (0.3 IU/kg/day; NovoNordisk, Buenos Aires, Argentina) was administered.

Glycemia (G), Total Cholesterol (TC), Triglycerides (Tg) and Liver Enzymes (Alkaline Phosphatase [AP], Glutamic Pyruvate Transaminase [GPT], Glutamic Oxalacetic Transaminase [GOT])

These determinations were carried out with commercial kits and automated kinetic method (Metrolab, Merck Autoanalyzer; Merck, Darmstadt, Germany), as described previously [12]. They were performed at the mentioned time points with the objective of evaluating if SOM230 affects glucose or lipid metabolism or if it can present hepatotoxicity. In the case that signs of diabetes mellitus, hyperlipidemia or hepatic dysfunction were suspected, analyses were performed at moments other than the ones mentioned in the protocol. In the case that any pathologies were encountered, medication was suspended and the dog was taken out of the study. Although hypercortisolism is expected at the time of diagnosis, with alterations in lipids (increase in cholesterol and/or triglycerides), glycemia and hepatic enzymes (principally AP and GPT) [27, 31], in the case of a decrease in cortisol concentrations (indicated by UCCR), the above-mentioned variables must decrease and/or become normal. In the event that ACTH and UCCR became normal, without Cushing's disease symptoms but with an increase in hepatic enzymes (triple increase of GPT with respect to the initial measurement and GOT doubled), the study subject was to be immediately removed from the study because it was considered a side effect of SOM230.

Cell Culture and Transfection

The mouse corticotropinoma cell line AtT-20 (American Type Culture Collection, Manassas, Va., USA) was used. Cells were cultured in DMEM supplemented with 10% FCS, 2 nmol/l glutamine, 0.5 mg/l partricin, and 10^5 U/l penicillin-streptomycin at 37°C and 5% CO_2. Cell culture materials were purchased from Life Technologies (Karlsruhe, Germany), Nunc (Wiesbaden, Germany), and Sigma (St. Louis, Mo., USA). Cell transfection was performed with SuperFect® (Qiagen GmbH, Hilden, Germany) as previously described [32]. The constructs containing the luciferase gene downstream to the POMC promoter, AP1, NurRE, NBRE, and the POMC promoter containing a mutant NurRE (POMC-Δ-NurRE) have been described elsewhere [11]. The Gβγ inhibitor, β-ARK-CT (gift of P. Voigt, Institute of Pharmacology, Charité Medical University, Campus Benjamin Franklin, Berlin, Germany), was used as previously described [32]. Double-stranded small interfering RNAs (siRNA) against mouse SSTR2 (agaccgagccauacuacgatt) and SSTR5 (uagugccugugcucuacuutt) were synthesized by MWG Biotech (Ebersberg, Germany) and one scrambled siRNA (Scramble II; MWG Biotech) was used as a control. Transfected cells were treated with 10^{-9} mol/l SOM230 for 6 h. When indicated, pertussis toxin (Sigma) was administered 12 h before SOM230 treatment. Luciferase activity was measured by Berthold luminometer (Berthold Detection Systems, Pforzheim, Germany) and transfection efficiency was determined using the pEGFP-C2 Vector (Clontech, Saint-Germain-en-Laye, France) which encodes an optimized variant of the green fluorescent protein (GFP). Data are expressed as the ratio of relative luciferase activity to GFP absorbance. Western blot was employed to confirm the successful knocking down of SSTR2 and SSTR5 using SSTR type-specific rabbit polyclonal antibodies (Gramsch Laboratories, Schwabhausen, Germany) (online suppl. fig. 1A, www.karger.com/doi/10.1159/000327429). In addition, successful silencing was confirmed by RT-PCR using specific primers for mouse SSTR2 (5′-cttggccatgcaggtggcgctagt-3′ and 5′-caatgatgtcttccgctccggatt-3′) and SSTR5 (5′-catgagtgtcgaccgctacc-3′ and 5′-ggcacagctattggcataag-3′) (online suppl. fig. 1B). RNA was extracted from the same cell pool that gave the lysates for the Western blot and cells for the assay, using TRIzol (Invitrogen, Carlsbad, Calif., USA). Each experiment was performed in duplicate.

Statistical Analysis

The analyses for ACTH, α-MSH, UCCR and weight variation were performed with the repeated measures ANOVA test, followed by a Dunnett's test (basal vs. analyzed time points) and the Bonferroni post-test to compare between averages. The linear trend was also calculated. Tumor size, clinical evaluation data (with the exception of weight), G, TC, Tg and liver enzymes (initial and final values) were compared using paired samples tests (paired t test or Wilcoxin U test, according to whether the distribution of the data was parametric or not). Results were expressed according to whether the distribution was parametric or not, as average ± SD or median and ranges, with a level of significance of 5% ($p < 0.05$). In cell culture experiments, differences after treatment were assessed by one-way ANOVA in combination with Scheffé's test, and p values <0.05 were considered significant.

Ethical Approval

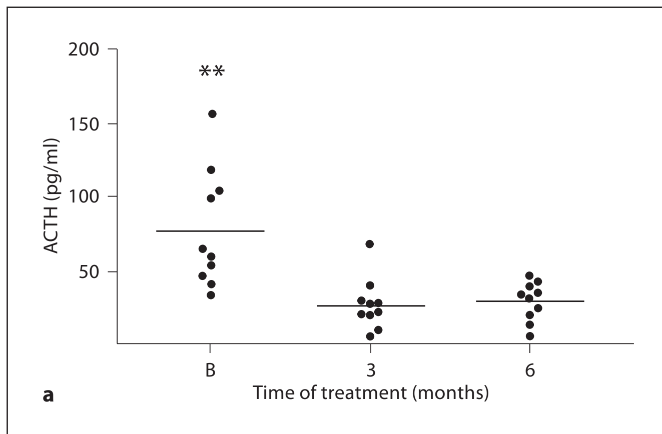
The project and study protocol were approved by the Ethics Committee of the Facultad de Ciencias Veterinarias, Universidad de Buenos Aires (CICUAL, UBACyT project V006), according to Argentine law and recommendations of the World Health Organization (WHO). This Ethics Committee approved the protocols considering each study subject as its own control, taking into account that the use of a control group treated with ketoconazole had already been carried out by us and is well known from previous studies [12, 19], and with the knowledge of the outcome of these studies, the use of a third group, as a control, treated with ketoconazole, was not justified. The dog owners signed a consent form to allow the administration of SOM230 and the performance of the laboratory tests.

Results

ACTH, α-MSH and UCCR in Both Test Groups

A significant decrease in ACTH was observed in both test groups at the designated test times when compared to baseline levels before treatment (B) (fig. 1). In TG1, the decrease was significant (ANOVA, p = 0.0006, F: 11.5), at 3 m ($p < 0.001$) and 6 m ($p < 0.01$) versus the value at the B time point, although there were no differences between 3 m and 6 m. ACTH was decreased by 48.12% at the end of the test period. The test for linear trend indicated that the decrease is linear and significant (p = 0.007, slope = –24.4, $R^2 = 0.34$) (fig. 1a).

In TG2, ACTH also diminished significantly (ANOVA, p = 0.01, F: 3.42) during the treatment at 2 m ($p < 0.05$), 6 m and 10 m ($p < 0.01$) versus B (cycles of SOM230 administration) and also at 4 and 8 m (rest periods) ver-



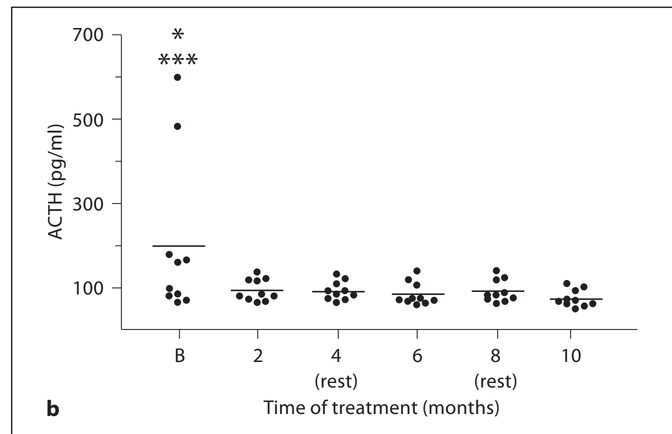


Fig. 1. Variation of ACTH in dogs with Cushing's disease treated with SOM230. Treatment group 1 (TG1, 0.03 mg/kg/12 h SOM230 continuously during 6 months) (**a**) and treatment group 2 (TG2, 0.03 mg/kg/12 h SOM230 for 2 months alternated with a 2-month rest period, for a total of 10 months) (**b**). Normal values of ACTH were considered to be up to 65 pg/ml, with normal UCCR values. In both groups, once ACTH decreases, it does not increase later in the treatment, even during the drug-arrest cycles in TG2. **a** ** $p < 0.01$ before treatment (B) versus 3 months (3 m) and 6 m. There are no significant differences between 3 and 6 m. **b** * $p < 0.05$ B versus 2, 4 and 8 m; *** $p < 0.001$ B versus 6 and 10 m. There are no significant differences between 2 and 10 m. Repeated measures ANOVA-Dunnett's test. The solid line indicates the average, each circle represents 1 dog treated with SOM230.

sus B ($p < 0.05$). In this treatment group, ACTH was decreased by 47.3% at the end of the test period. No statistically significant differences were proven between the cycles of treatment with SOM230 nor between the rest periods. The test for linear trend also proved that the decrease was linear and significant ($p = 0.0036$, slope = –9.13, $R^2 = 0.13$) (fig. 1b).

There were no significant changes in α-MSH during treatment in either of the two test groups (data not shown). Only 1 dog in TG1 and 2 dogs in TG2 had decreases, to normal values of α-MSH, while the rest of the dogs had constant values during the study period.

UCCR also diminished significantly in both treatment groups (fig. 2). In TG1, the decrease was significant (ANOVA, $p < 0.0001$, F: 30.7) at 3 and 6 m ($p < 0.001$) versus B, and there were no differences between 3 and 6 m. UCCR dropped 47% by the end of the test period. The test for linear trend was also significant ($p < 0.0001$, slope = –21.8, $R^2 = 0.55$) (fig. 2a).

In TG2, a significant reduction (ANOVA, $p = 0.0005$, F: 5.31) between B versus 2 m ($p < 0.05$) and among B versus 6, 8 and 10 m ($p < 0.01$) were observed. There were no significant differences between B versus 4 m. In this treatment group, UCCR dropped by 73.3% by the end of the test period. There are differences among the 4 months (rest periods) versus 6 and 10 m ($p < 0.05$) of treatment with SOM230, because a tendency of UCCR increase can be observed in most dogs during the rest periods. The test for linear trend was significant in TG2 ($p < 0.0001$, slope = –15.8, $R^2 = 0.18$) (fig. 2b).

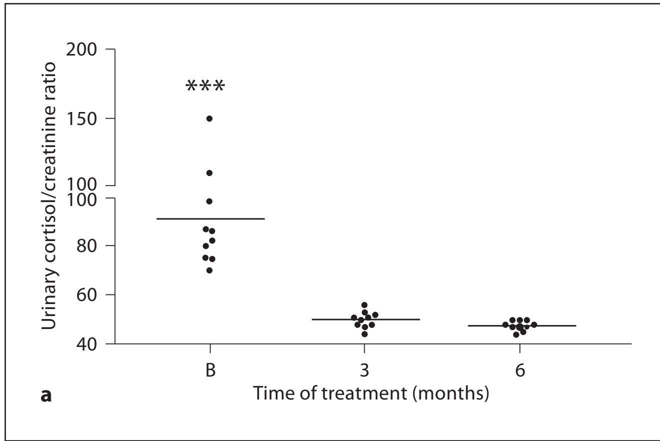
Overall, in TG1, 10/10 dogs obtained ACTH and UCCR values within the reference range by the end of the treatment. In TG2, 7/10 dogs reached normal values, while the remaining 3 had values slightly above the reference range, although lower than the ones they had at B. Additionally, considering that these 3 dogs had particularly large tumors at the beginning of the treatment, and the fact that their UCCR values were within the normal range at the end of the treatment, it is likely, although not proven, that SOM230 had a positive effect on ACTH and UCCR also in these 3 dogs.

NMRI Analysis of the Treatment Groups

In both treatment groups, the size of the adenomas significantly decreased at the end of the treatment, with $p = 0.04$ at 6 m versus B in TG1 (fig. 3a), while in TG2 (fig. 3b) the difference is $p = 0.002$ at 10 m versus B. The paramagnetic signal intensity is high at the B time point, and it becomes isointense (same intensity as the rest of the encephalon) at the end of the treatment in both groups, including dogs that did not show a reduction in tumor size (fig. 4).

Clinical Signs, G, TC, Tg and Liver Enzymes

The clinical signs improved towards the end of the treatment in both groups. The weight in TG1 reduced sig-



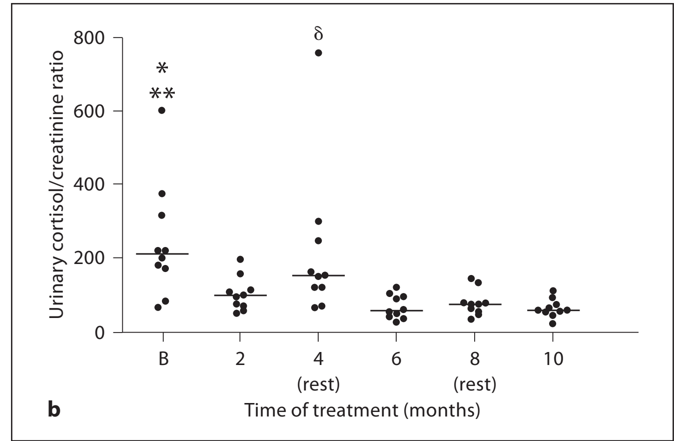
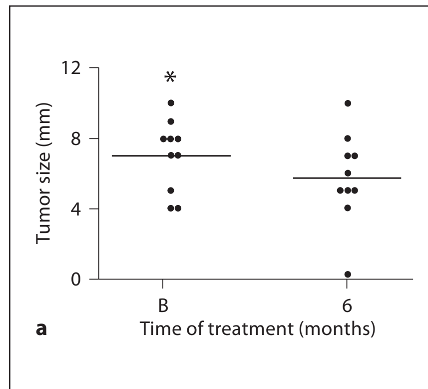


Fig. 2. Variations in UCCR in dogs with Cushing's disease treated with SOM230. Treatment group 1 (TG1, 0.03 mg/kg/12 h SOM230 continuously during 6 months) (**a**) and treatment group 2 (TG2, 0.03 mg/kg/12 h SOM230 for 2 months alternated with a 2-month rest period, for a total of 10 months) (**b**). Normal values of UCCR were considered to be up to 45 μg/dl. As occurs with ACTH levels, UCCR levels diminish, and once they do, they do not increase later during the treatment, even during the drug-arrest cycles in TG2. **a** *** $p < 0.001$ before treatment (B) versus 3 and 6 months. There are no significant differences between 3 and 6 m. **b** * $p < 0.05$ B versus 2 m and ** $p < 0.01$ B versus 6, 8 and 10 m. There are no significant differences between B and 4 m. Difference (δ): $p < 0.05$, 4 m versus 6, 8 and 10 m. Repeated measures ANOVA-Dunnett's test and Bonferroni's test. The solid line indicates the average, each circle represents 1 dog treated with SOM230.



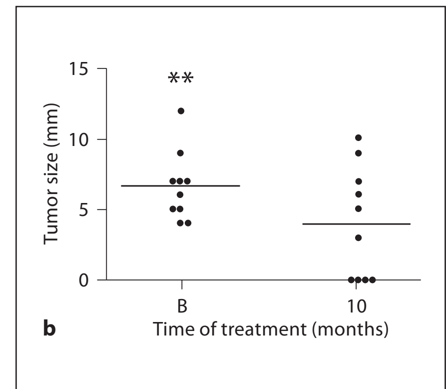


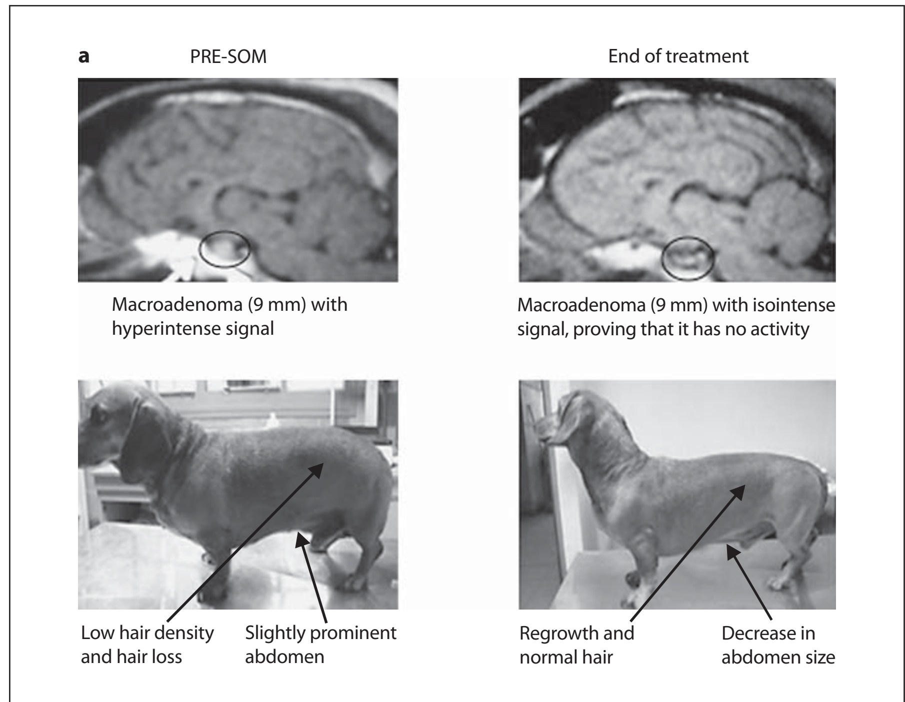
Fig. 3. Tumor size evaluated through NMRI in dogs with Cushing's disease treated with SOM230. Treatment group (TG1, 0.03 mg/kg/12 h SOM230 continuously during 6 months) (**a**) and treatment group 2 (TG2, 0.03 mg/kg/12 h SOM230 for 2 months alternated with a 2-month rest period, for a total of 10 months) (**b**). In the case of non-detectable tumors, the pituitary's aspect in NMR images was normal and isointense, and a size of 0 mm was assigned. **a** * $p = 0.04$ before treatment (B) versus 6 months (6 m). **b** ** $p = 0.002$ B versus 10 m. Paired t test. The solid line indicates the average.

nificantly (ANOVA, $p = 0.05$, F: 3.5), with differences ($p < 0.05$) between the weight at the beginning of the treatment (14.3 ± 6.6 kg) versus 6 m (13.2 ± 5.8 kg), with a trend to decrease significantly ($p = 0.025$, slope = −0.6, $R^2 = 0.007$). In TG2, the decrease is significant (ANOVA, $p = 0.0003$, F: 5.8), with differences between B (9 ± 3.6 kg) versus 8 m (8.3 ± 3.2 kg, $p < 0.05$) and versus 10 m (8.1 ± 3.1 kg, $p < 0.01$).

The estrous cycle returned in 6 (3 in each group) out of the 10 non-neutered females. The gonadal axis activity was also recovered between 3 and 5 months of treatment.

The rest of the evaluated clinical signs presented significant improvement in both treatment groups (fig. 5), particularly with respect to fluid uptake and micturition frequency, skin aspect and hair, and reduction in abdomen size.

Regarding glucose (G) (table 1), 3 dogs in TG1 developed diabetes at 4 months of treatment, although it became controlled with low dosis of insulin detemir (0.3 IU/kg/day); therefore, at 6 m glycemia values (100.9 ± 18.8 mg/dl) did not present significant differences versus basal (91 ± 13.6 mg/dl). We cannot conclude that the diabetes is due to SOM, as will be discussed below. In TG2 there



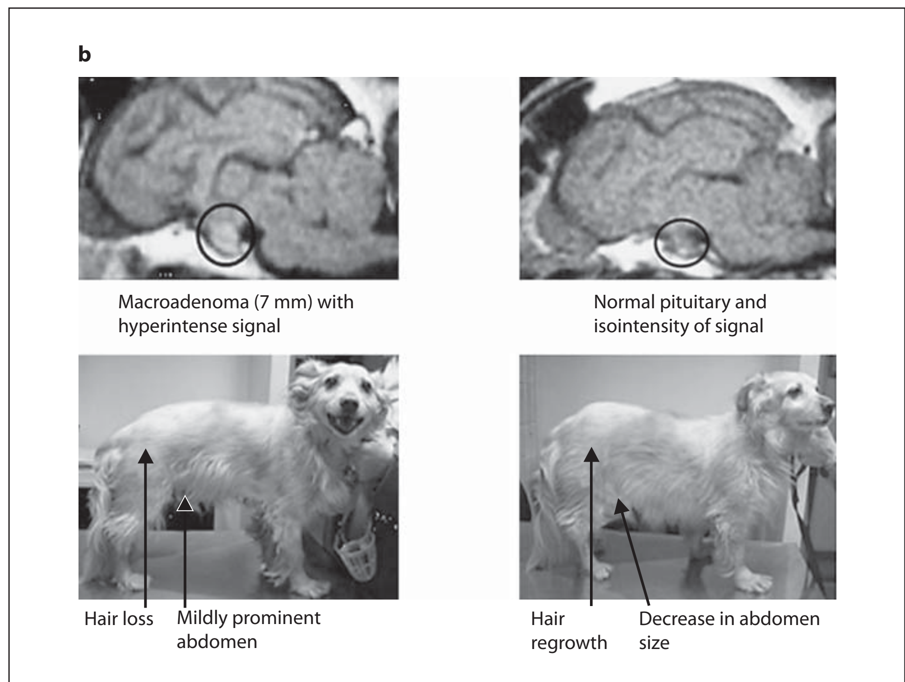


Fig. 4. Example of clinical progress and changes in tumor size and paramagnetic signals in dogs with Cushing's disease treated with SOM230. Treatment group (TG1, 0.03 mg/kg/12 h SOM230 continuously during 6 months) (**a**) and treatment group 2 (TG2, 0.03 mg/kg/12 h SOM230 for 2 months alternated with a 2-month rest period, for a total of 10 months) (**b**). **a** Note: although the tumor maintains its large size, the paramagnetic signal is isointense. **b** On the contrary, tumor size reduction can be clearly seen.

were no significant differences during the treatment (whether between dogs of the same group or individually), and it maintained itself within the reference values for dogs. TC (table 1) did not vary significantly during the treatment in both groups. Tg (table 1) decreased significantly in TG1 ($p < 0.01$) at 6 m versus B. There were no significant differences in TG2 between 10 m versus B.

Among the liver enzymes analyzed (table 1), AP did not suffer significant variations in TG1, although a trend towards a decrease can be observed at 6 m versus B. In

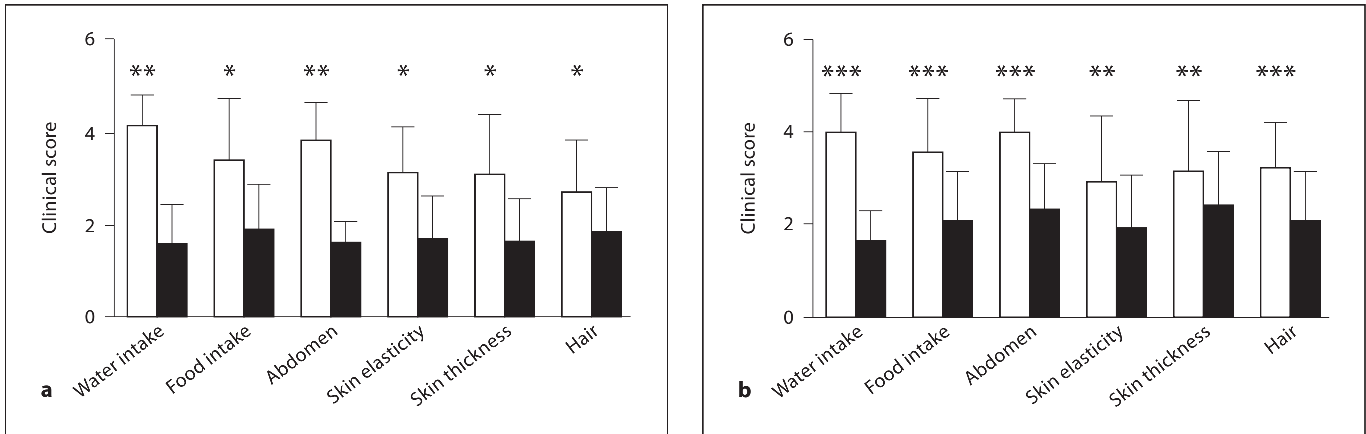


Fig. 5. Assessment of clinical signs in dogs with Cushing's disease treated with SOM230. Treatment group 1 (TG1, 0.03 mg/kg/12 h SOM230 continuously during 6 months) (**a**) and treatment group 2 (TG2, 0.03 mg/kg/12 h SOM230 for 2 months alternated with a 2-month rest period, for a total of 10 months) (**b**), at the end of treatment with SOM230. **a** * $p < 0.05$; ** $p < 0.01$ before treatment (B) versus 6 months (6 m). **b** ** $p < 0.01$; *** $p < 0.001$ B versus 10 m. White bars correspond to values at B, black bars correspond to the values at the end of treatment. Paired t test, data expressed as average ± SD.

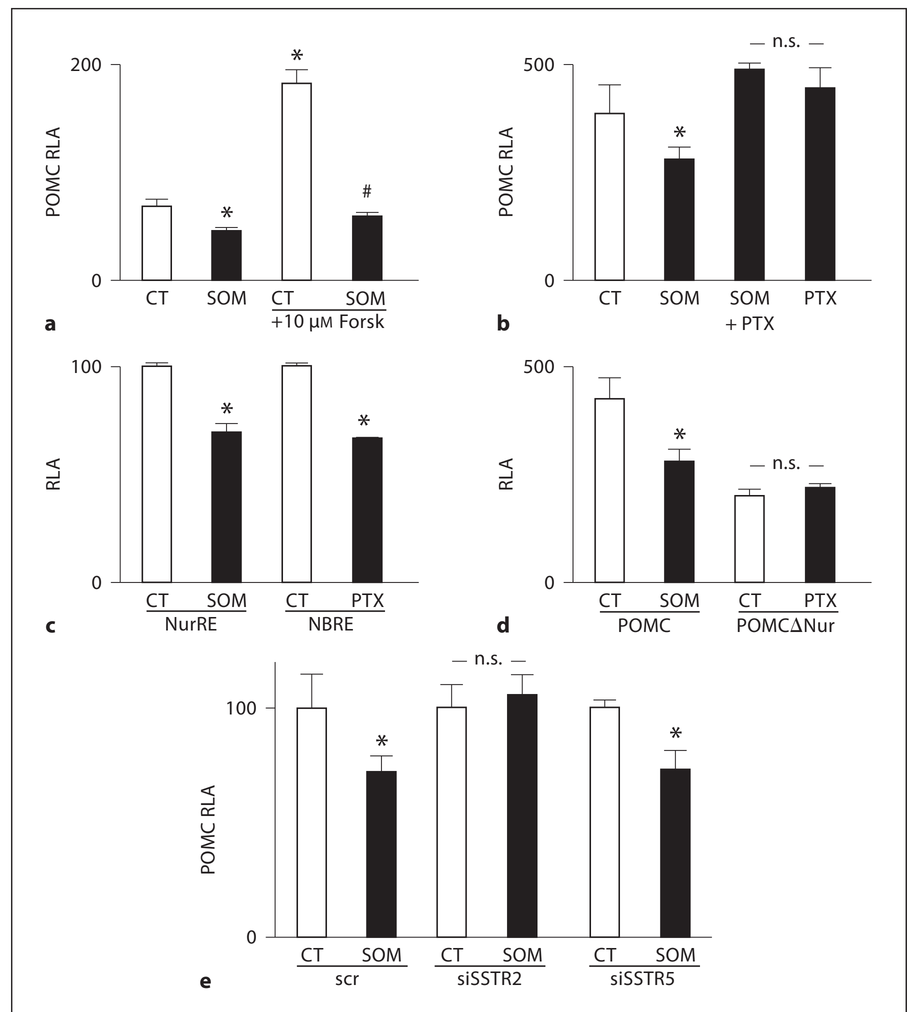


Fig. 6. Transcriptional action of SOM230 in AtT20 corticotropic cells. Effect of 6 h treatment with 1 nM SOM230 (SOM) in AtT20 corticotropic cells: vehicle (CT) or forskolin (Forsk)-treated cells (**a**) and after 16 h pretreatment with 100 ng/ml pertussis toxin (PTX) (**b**), on POMC promoter activity in AtT-20 cells transfected with POMC-Luc. Effect of 6 h treatment with 1 nM SOM230 on Nur transcriptional activity in AtT-20 cells transfected with NurRE and NBRE (**c**) and on POMC promoter bearing a mutation in the Nur77/Nurr1-binding site (POMCΔNur) (**d**). The effect of SSTR2 and SSTR5 RNA interference on SOM230 action was determined: POMC promoter activity was measured in AtT-20 cells transfected with scramble, 100 nM SSTR2- or SSTR5-siRNA. Data are presented as percentage of each individual control (**e**). Results are shown as relative luciferase activity (RLA): GFP ratio. Each experiment was repeated twice. All treatments were performed in serum-free DMEM. * $p < 0.05$ to vehicle control (CT). $^{\#}$ $p < 0.05$ to forskolin (Forsk) treatment.

Table 1. Analysis of glycemia (G), total cholesterol (TC), triglycerides (Tg) and liver enzymes (alkaline phosphatase [AP], glutamic pyruvate transaminase [GPT], glutamic oxalacetic transaminase [GOT]) in dogs with Cushing's disease treated with SOM230

	Treatment group 1		Treatment group 2	
	before treatment	6 m	before treatment	10 m
G, mg/dl	91 ± 13.6	100.9 ± 18.8	82.9 ± 18.7	77.1 ± 18.8
TC, mg/dl	277 ± 39.2	259.1 ± 80.7	277 ± 74.7	283 ± 56.5
Tg, mg/dl	170.5 (55–333)	116 (21–200)**	81 (33–563)	90 (24–226)
AP, IU/l	1,025 (305–3,050)	441 (205–1,521)	463 (50–3,100)	195.2 (15–1,500)**
GPT, IU/l	118 (57–168)	87 (20–120)*	97 (35–240)	66.5 (17–140)**
GOT, IU/l	55 (21–84)	34 (21–79)	51.5 (17–138)	30.5 (20–80)

Treatment group 1 (TG1, 0.03 μg/kg/12 h SOM230 continuously during 6 months) and treatment group 2 (TG2, 0.03 μg/kg/12 h SOM230 for 2 months alternated with a 2-month rest period, for a total of 10 months).

* $p < 0.05$ and ** $p < 0.01$ versus their respective values before treatment.

Tg, AP, GPT and GOT are expressed as medians and ranges, compared using Wilcoxin U test. Normal reference ranges: G: 60–110 mg/dl; TC: up to 250 mg/dl; Tg: up to 150 mg/dl; AP up to 300 IU/l; GPT and GOT up to 80 IU/l.

TG2 there is a significant decrease ($p < 0.01$) between 10 m versus B.

GPT significantly dropped in both TG1 ($p < 0.05$) and TG2 ($p < 0.01$) versus their respective B. Analyzing each animal in each group individually, in TG1, 6/10 animals already had considerably high levels of GPT, while at 6 m only 3/10 had slightly higher levels than the cut-off value (80 IU/l). In TG2, 6/10 dogs presented high levels of GPT, with only 2 dogs having a slightly higher level than the cut-off value. GOT did not present significant variations in either treatment group, being maintained within the range of the reference limits for the method used (up to 80 IU/l), towards the end of the study. Performing an individual analysis, 3/10 dogs of TG2 had high levels of this enzyme at the beginning of the study and later decreased to the reference range.

Transcriptional Action of SOM230 on ACTH Synthesis

The mechanism of SOM230 action on ACTH synthesis was investigated in AtT-20 immortalized mouse corticotropinoma cells, which express SSTR2 and SSTR5 [23]. Treatment with SOM230 inhibited the basal and forskolin-induced activity of the POMC promoter (fig. 6a). Preincubation with 100 ng/ml pertussis toxin for 12 h abolished the inhibitory effect of SOM230, indicating an inhibitory G protein (G_i)-mediated effect (fig. 6b). SOM230 action was not abolished in cells transfected with β-ARK-CT, revealing the involvement of the G_i α-subunit, but not of the βγ dimers (data not shown). SOM230 treatment had no effect on basal or forskolin-induced AP1 transcriptional activity (data not shown), but it inhibited basal (fig. 6c) and forskolin-induced NurRE (Nur77/Nurr1 dimer-dependent) and NBRE (Nur77 or Nurr1 monomer-dependent) transcriptional activities. Furthermore, SOM230 did not affect a POMC promoter with a mutant NurRE (fig. 6d), indicating that the drug's inhibitory action is mediated by the suppression of the Nur family of transcription factors. SSTR2 knockdown by RNA interference completely abolished the SOM230 effect on POMC (fig. 6e). In contrast, SOM230 suppressed POMC promoter activity in AtT-20 cells transfected with SSTR5 siRNA.

Discussion

SOM230 was capable of controlling, during the study period, Cushing's disease in dogs, without causing severe adverse side effects. This can be clearly seen with the decrease in ACTH in both treatment groups, accompanied by the reduction in adrenal cortisol evaluated by the UCCR method. It is of interest that drug suspension for 2 months in TG2 did not result in hormone increase back to their pretreatment values (B), allowing the assumption of a sustained or residual effect of SOM230. In TG1, all of the dogs showed improvement, while in TG2, 7/10 completely responded, while the remaining 3 partially re-

sponded, having no clinical manifestation of Cushing's disease, but ACTH values that were not optimal, although improved. In this group, it can be observed that when SOM230 treatment was interrupted, there was a slight increase in UCCR levels. Possibly, continuous treatment with SOM230, as opposed to an intermittent one, might have a more prolonged, and therefore more effective, outcome on corticotropinoma cells.

Although it is described that SSTR2, as well as the other receptor isoforms, can desensitize to somatostatin analog action [33, 34], we have not observed, at least during the treatment period, escape from SOM230 effect. Cycles of drug treatment alternating with treatment-free periods might prevent SSTR desensitization. Performing a 6-month follow-up of the dogs after the study, these did not manifest recurrences typical of Cushing's disease up to the moment. POMC transcription also normalized, as reflected by the decrease in α-MSH in the 3 cases in which it was initially high (1 dog in TG1 and 2 dogs in TG2). Although ACTH and α-MSH secretion is equimolar in most dogs, those with large-sized adenomas have greater levels of α-MSH [28, 35]. The 3 dogs with the initially elevated α-MSH secretion had macroadenomas and responded adequately to SOM230 drug action. Additionally, it is described that tumor size in dogs correlates with ACTH precursor levels as well as with lack of inhibition by dexamethasone [35, 36]. It might be assumed that SSTR2 is still expressed, at least in dogs, even in tumors of large size or of aggressive molecular characteristics. Indeed, a recent study showed that in contrast to humans, canine corticotropinomas predominantly express SSTR2 [24]. Possibly, the reported resistance to inhibition by dexamethasone in dogs might be one of the causes by which SSTR2 is not only downregulated, but even overexpressed. The previously reported high expression of SSTR2 in SOM230-responsive canine corticotropinomas implies that this receptor subtype most probably mediates the drug's inhibitory action on ACTH. SSTR2 and SSTR5 are the receptor subtypes shown to downregulate ACTH in AtT-20 mouse corticotropinoma cells [37]. AtT-20 cells predominantly express SSTR2 and SSTR5 [23], providing a good model to study the mechanism behind the inhibitory action of SOM230 on POMC products. Indeed in this cell model, SOM230 was found to mediate its suppressive action on POMC promoter exclusively through the SSTR2, since knocking down this receptor completely abolished the drug's effect. These data reveal a novel mechanism by which SSTR2 contributes to SOM230's antisecretory action. Taking this into account, and the preferred expression of SSTR2 in dogs is suggested by strong mRNA expression measured by qPCR and the immunohistochemical detection of SSTR2, the rationale for future studies using octreotide for the treatment of canine corticotropinoma is supported and in line with the results presented by de Bruin et al. [24].

POMC transcription is under the control of AP1 and the nuclear orphan receptors Nur77 and Nurr1 [38]. SOM230 suppresses cAMP and potently inhibits hormone secretion. In the present study, we show that SOM230 is also able to suppress POMC transcription by decreasing Nur77 and Nurr1 homo- and heterodimer transcriptional activities. The importance of this mechanism is highlighted by the fact that SOM230 cannot suppress the POMC promoter bearing a mutation in the Nur77/Nurr1-binding site. SOM230's suppressive effect on POMC promoter was pertussis toxin sensitive but was not abolished after sequestering the βγ subunits by the β-ARK-CT, indicating the involvement of only the α-subunit, which inhibits adenylate cyclase and attenuates cAMP accumulation. Nur77 and Nurr1 are upregulated downstream to the cAMP/PKA pathway [39], therefore this activation of the G_i α-subunit may lead to the decreased Nur77/Nurr1 transcriptional activity observed in SOM230-treated AtT-20 cells. Therefore, in AtT-20 cells, SOM230 acting through SSTR2 suppresses POMC promoter through a mechanism involving the G_i α-subunit and decreased Nur77/Nurr1 transcriptional activity on the POMC promoter. Altogether, these data provide a novel mechanism in which SOM230 inhibits POMC promoter by downregulating cAMP-mediated Nur77/Nurr1 transcription.

Interestingly, a recent publication showed that somatostatin analogs regulate ACTH synthesis through bone morphogenetic protein 4 (BMP-4) [40]. The BMP system plays a crucial role in pituitary tumorigenesis [7, 41, 42], and therefore it is possible that changes in components of this system after somatostatin analog treatment downstream to G_i α-subunit are also involved in the transcriptional changes described in the present study. Despite the fact that the AtT20 mouse corticotropic cell line possesses differences in comparison with canine corticotropinomas, the results presented here allow an insight in some detail upon the molecular mechanisms in which this drug exerts its actions, and suggests a possible mechanism through which it exerts its therapeutic effects.

The resistance that corticotropic cells develop towards cortisol has been indicated as one of the causes of corticotropinoma development and its aggressive behavior [5, 43]. In our study we have seen that adenoma size, as well

as ACTH and cortisol levels, were significantly reduced in both treatment groups, although in some animals studied whose tumors were >7 mm, there was no evidence of size decrease, but there was no increase either, and there were variations in the paramagnetic signal intensities, becoming isointense. With regard to the basal values of ACTH and UCCR, at the beginning of the study, there are greater values of ACTH and UCCR in the animals that belonged to TG2 than those in TG1. This could possibly be explained by the fact that the animals in each TG entered the study during different periods in the year, in particular different photoperiods. Most (7/10) dogs in TG1 entered the study during a decreasing photoperiod, while the majority in TG2 (6/10) entered the study during an increasing photoperiod. It has been observed in other mammal species that cortisol and the corticotropic area, as well as the sensitivity of the adrenals to ACTH, vary with photoperiod [44, 45]. SOM230 was found to inhibit tumor growth and reduce tumor size in vitro and in vivo [reviewed in 25]. SSTR2 blocks cell cycle progression by activating phosphotyrosine phosphatases and affecting the MAPK and PI3K/Akt signaling pathways [reviewed in 46]. In addition, SSTR2 and SSTR3 were shown to induce apoptosis [47, 48]. In the present study, the antimitogenic action of SOM230 was evident in the majority of the cases analyzed. In those cases where size reduction did not occur, the tumor did not increase in size and its activity was inhibited, as demonstrated by the isointense paramagnetic signals and the reduction in ACTH and UCCR.

Clinical improvement was evident, having as most prominent sign the recovery of the estrous cycle after normalization of cortisol levels, where cortisol acts as an inhibitor of the gonadotropic axis [49]. We have not observed side effects induced by SOM230 treatment, and although possible insulin inhibition and the inherent risk of diabetes have been described [50, 51], these did not occur, with the exception of 3 dogs, in which glycemia became normal after treatment with low doses of insulin detemir. Nevertheless, it is not ascertained that the hyperglycemia in these animals was caused by SOM230 treatment, because in our observations, 12–15% of dogs that suffer of Cushing's disease develop diabetes independently of the treatment they receive, while 1–2 out of 10 dogs that come to the veterinary clinic to consult for Cushing's disease already have diabetes once the diagnosis of Cushing's is confirmed [Castillo, unpubl. data], as described by others as well [52]. Evaluating glycemia 1–2 h post-treatment with SOM230 in 3 dogs, we did not observe acute increases nor did we see it in long-term treatment with the drug. In some cases, the diabetes of some dogs was due to the fact they had Cushing's disease, even though at the time of the diagnosis of diabetes it was not known they had Cushing's. In these animals, cortisol produces an anti-insulinic effect (inhibition and exhaustion of pancreatic β cells, inhibition of glucose uptake by cells in the periphery, increase of gluconeogenesis in the liver), explaining the development of diabetes. Unlike humans, the fact that canine blood sugar values do not seem to be altered with SOM230 treatment leads to believe that this drug does not affect their pancreatic β cells and insulin secretion. Alternatively it might be that an inhibitory effect on insulin secretion is compensated by an even stronger inhibitory effect on glucagon secretion and thus there would be no net effect on plasma glucose. In general, the apparent differences of SOM230 on glucose homeostasis in humans and dogs can be due to species differences, which have been previously observed, e.g. between monkeys and rats where only rats show a transient hyperglycemia in response to SOM230.

Regarding the liver enzymogram, SOM230 did not induce liver damage. The fact that GPT levels decreased and GOT levels did not increase, even dropping in those dogs in which it was initially elevated, is an indicator that SOM230 treatment is safe in this respect.

The present study demonstrates that SOM230 suppresses hormone secretion and POMC transcription and ameliorates Cushing's disease symptoms in dogs. SOM230 has a potent antisecretory action on ACTH, which is primarily mediated through SSTR5 in humans [23]. To which extent the strong inhibitory effect of SOM230 on ACTH secretion in dogs is also mediated by SSTR5 remains to be seen once dog-specific antibodies against SSTR5 are available. It is, however, also likely that additional activation of SSTR1, 2, and 3 receptor subtypes contribute the effect of SOM230 in dogs. In addition, the suppressive effect of SOM230 on POMC transcription through SSTR2 described herein suggests that this beneficial effect could also contribute to the efficacy of SOM230 in human patients with Cushing's disease.

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References

1 Swearingen B, Biller BM, Barker FG 2nd, Katznelson L, Grinspoon S, Klibanski A, Zervas NT: Long-term mortality after transsphenoidal surgery for Cushing disease. Ann Intern Med 1999;130:821–824.
2 Morris D, Grossman A: The medical management of Cushing's syndrome. Ann NY Acad Sci 2002;970:119–133.
3 Pereira AM, van Aken MO, van Dulken H, Schutte PJ, Biermasz NR, Smit JW, Roelfsema F, Romijn JA: Long-term predictive value of postsurgical cortisol concentrations for cure and risk of recurrence in Cushing's disease. J Clin Endocrinol Metab 2003;88:5858–5864.
4 Dahia PL, Grossman AB: The molecular pathogenesis of corticotroph tumors. Endocr Rev 1999;20:136–155.
5 Lamberts SW: Glucocorticoid receptors and Cushing's disease. Mol Cell Endocrinol 2002;197:69–72.
6 Melmed S: Mechanisms for pituitary tumorigenesis: the plastic pituitary. J Clin Invest 2003;112:1603–1618.
7 Giacomini D, Paez-Pereda M, Theodoropoulou M, Labeur M, Refojo D, Gerez J, Chervin A, Berner S, Losa M, Buchfelder M, Renner U, Stalla GK, Arzt E: Bone morphogenetic protein-4 inhibits corticotroph tumor cells: involvement in the retinoic acid inhibitory action. Endocrinology 2006;147:247–256.
8 Levesque D, Rouillard C: Nur77 and retinoid × receptors: crucial factors in dopamine-related neuroadaptation. Trends Neurosci 2007;30:22–30.
9 Teshima T, Hara Y, Takekoshi S, Teramoto A, Osamura RY, Tagawa M: Expression of genes related to corticotropin production and glucocorticoid feedback in corticotroph adenomas of dogs with Cushing's disease. Domest Anim Endocrinol 2009;36:3–12.
10 Labeur M, Arzt E, Stalla GK, Paez-Pereda M: New perspectives in the treatment of Cushing's syndrome. Curr Drug Targets Immune Endocr Metabol Disord 2004;4:335–342.
11 Paez-Pereda M, Kovalovsky D, Hopfner U, Theodoropoulou M, Pagotto U, Uhl E, Losa M, Stalla J, Grubler Y, Missale C, Arzt E, Stalla GK: Retinoic acid prevents experimental Cushing syndrome. J Clin Invest 2001;108:1123–1131.
12 Castillo V, Giacomini D, Paez-Pereda M, Stalla J, Labeur M, Theodoropoulou M, Holsboer F, Grossman AB, Stalla GK, Arzt E: Retinoic acid as a novel medical therapy for Cushing's disease in dogs. Endocrinology 2006;147:4438–4444.
13 Pivonello R, Ferone D, de Herder WW, Kros JM, De Caro ML, Arvigo M, Annunziato L, Lombardi G, Colao A, Hofland LJ, Lamberts SW: Dopamine receptor expression and function in corticotroph pituitary tumors. J Clin Endocrinol Metab 2004;89:2452–2462.
14 Hofland LJ, Lamberts SW: The pathophysiological consequences of somatostatin receptor internalization and resistance. Endocr Rev 2003;24:28–47.
15 Van der Hoek J, Waaijers M, van Koetsveld PM, Sprij-Mooij D, Feelders RA, Schmid HA, Schoeffter P, Hoyer D, Cervia D, Taylor JE, Culler MD, Lamberts SW, Hofland LJ: Distinct functional properties of native somatostatin receptor subtype 5 compared with subtype 2 in the regulation of ACTH release by corticotroph tumor cells. Am J Physiol Endocrinol Metab 2005;289:E278–E287.
16 Batista DL, Zhang X, Gejman R, Ansell PJ, Zhou Y, Johnson SA, Swearingen B, Hedley-Whyte ET, Stratakis CA, Klibanski A: The effects of SOM230 on cell proliferation and adrenocorticotropin secretion in human corticotroph pituitary adenomas. J Clin Endocrinol Metab 2006;91:4482–4488.
17 Colao A, Lombardi G, Annunziato L: Cabergoline. Expert Opin Pharmacother 2000;1:555–574.
18 Shraga-Slutzky I, Shimon I, Weinshtein R: Clinical and biochemical stabilization of nelson's syndrome with long-term low-dose cabergoline treatment. Pituitary 2006;9:151–154.
19 Castillo VA, Gomez NV, Lalia JC, Cabrera Blatter MF, Garcia JD: Cushing's disease in dogs: cabergoline treatment. Res Vet Sci 2008;85:26–34.
20 Ambrosi B, Bochicchio D, Fadin C, Colombo P, Faglia G: Failure of somatostatin and octreotide to acutely affect the hypothalamic-pituitary-adrenal function in patients with corticotropin hypersecretion. J Endocrinol Invest 1990;13:257–261.
21 Stalla GK, Brockmeier SJ, Renner U, Newton C, Buchfelder M, Stalla J, Muller OA: Octreotide exerts different effects in vivo and in vitro in Cushing's disease. Eur J Endocrinol 1994;130:125–131.
22 Hofland LJ, van der Hoek J, van Koetsveld PM, de Herder WW, Waaijers M, Sprij-Mooij D, Bruns C, Weckbecker G, Feelders R, van der Lely AJ, Beckers A, Lamberts SW: The novel somatostatin analog SOM230 is a potent inhibitor of hormone release by growth hormone- and prolactin-secreting pituitary adenomas in vitro. J Clin Endocrinol Metab 2004;89:1577–1585.
23 Hofland LJ, van der Hoek J, Feelders R, van Aken MO, van Koetsveld PM, Waaijers M, Sprij-Mooij D, Bruns C, Weckbecker G, de Herder WW, Beckers A, Lamberts SW: The multi-ligand somatostatin analogue SOM230 inhibits ACTH secretion by cultured human corticotroph adenomas via somatostatin receptor type 5. Eur J Endocrinol 2005;152:645–654.
24 De Bruin C, Hanson JM, Meij BP, Kooistra HS, Waaijers AM, Uitterlinden P, Lamberts SW, Hofland LJ: Expression and functional analysis of dopamine receptor subtype 2 and somatostatin receptor subtypes in canine Cushing's disease. Endocrinology 2008;149:4357–4366.
25 Schmid HA: Pasireotide (SOM230): development, mechanism of action and potential applications. Mol Cell Endocrinol 2008;286:69–74.
26 Robben JH, van den Brom WE, Mol JA, van Haeften TW, Rijnberk A: Effect of octreotide on plasma concentrations of glucose, insulin, glucagon, growth hormone, and cortisol in healthy dogs and dogs with insulinoma. Res Vet Sci 2006;80:25–32.
27 Willeberg P, Priester WA: Epidemiological aspects of clinical hyperadrenocorticism in dogs (canine Cushing's syndrome). J Am Anim Hosp Assoc 1982;18:717–724.
28 Gallelli MF, Cabrera Blatter MF, Castillo V: A comparative study by age and gender of the pituitary adenoma and ACTH and α-MSH secretion in dogs with pituitary-dependent hyperadrenocorticism. Res Vet Sci 2010;88:33–40.
29 Weckbecker G, Briner U, Lewis I, Bruns C: SOM230: a new somatostatin peptidomimetic with potent inhibitory effects on the growth hormone/insulin-like growth factor-I axis in rats, primates, and dogs. Endocrinology 2002;143:4123–4130.
30 Meric S: Textbook of Veterinary Internal Medicine, ed 4. Philadelphia, Saunders, 1995.
31 Ling GV, Stabenfeldt GH, Comer KM, Gribble DH, Schechter RD: Canine hyperadrenocorticism: pretreatment clinical and laboratory evaluation of 117 cases. J Am Vet Med Assoc 1979;174:1211–1215.
32 Theodoropoulou M, Zhang J, Laupheimer S, Paez-Pereda M, Erneux C, Florio T, Pagotto U, Stalla GK: Octreotide, a somatostatin analogue, mediates its antiproliferative action in pituitary tumor cells by altering phosphatidylinositol 3-kinase signaling and inducing Zac1 expression. Cancer Res 2006;66:1576–1582.
33 Engström M, Savola JM, Wurster S: Differential efficacies of somatostatin receptor agonists for G-protein activation and desensitization of somatostatin receptor subtype 4-mediated responses. J Pharmacol Exp Ther 2006;316:1262–1268.
34 Holliday ND, Tough IR, Cox HM: A functional comparison of recombinant and native somatostatin sst2 receptor variants in epithelia. Br J Pharmacol 2007;152:132–140.
35 Bosje JT, Rijnberk A, Mol JA, Voorhout G, Kooistra HS: Plasma concentrations of ACTH precursors correlate with pituitary size and resistance to dexamethasone in dogs with pituitary-dependent hyperadrenocorticism. Domest Anim Endocrinol 2002;22:201–210.

36 Kooistra HS, Voorhout G, Mol JA, Rijnberk A: Correlation between impairment of glucocorticoid feedback and the size of the pituitary gland in dogs with pituitary-dependent hyperadrenocorticism. J Endocrinol 1997; 152:387–394.

37 Strowski MZ, Dashkevicz MP, Parmar RM, Wilkinson H, Kohler M, Schaeffer JM, Blake AD: Somatostatin receptor subtypes 2 and 5 inhibit corticotropin-releasing hormone-stimulated adrenocorticotropin secretion from AtT-20 cells. Neuroendocrinology 2002;75:339–346.

38 Therrien M, Drouin J: Pituitary pro-opiomelanocortin gene expression requires synergistic interactions of several regulatory elements. Mol Cell Biol 1991;11:3492–3503.

39 Kovalovsky D, Refojo D, Liberman AC, Hochbaum D, Pereda MP, Coso OA, Stalla GK, Holsboer F, Arzt E: Activation and induction of NUR77/NURR1 in corticotrophs by CRH/cAMP: involvement of calcium, protein kinase A, and MAPK pathways. Mol Endocrinol 2002;16:1638–1651.

40 Tsukamoto N, Otsuka F, Miyoshi T, Yamanaka R, Inagaki K, Yamashita M, Otani H, Takeda M, Suzuki J, Ogura T, Iwasaki Y, Makino H: Effects of bone morphogenetic protein on adrenocorticotropin production by pituitary corticotrope cells: involvement of up-regulation of bmp receptor signaling by somatostatin analogs. Endocrinology 2010;151:1129–1141.

41 Paez-Pereda M, Giacomini D, Refojo D, Nagashima AC, Hopfner U, Grubler Y, Chervin A, Goldberg V, Goya R, Hentges ST, Low MJ, Holsboer F, Stalla GK, Arzt E: Involvement of bone morphogenetic protein-4 in pituitary prolactinoma pathogenesis through a Smad/estrogen receptor crosstalk. Proc Natl Acad Sci USA 2003;100:1034–1039.

42 Giacomini D, Acuna M, Gerez J, Nagashima AC, Silberstein S, Paez-Pereda M, Labeur M, Theodoropoulou M, Renner U, Stalla GK, Arzt E: Pituitary action of cytokines: focus on BMP-4 and gp130 family. Neuroendocrinology 2007;85:94–100.

43 Karl M, Von Wichert G, Kempter E, Katz DA, Reincke M, Monig H, Ali IU, Stratakis CA, Oldfield EH, Chrousos GP, Schulte HM: Nelson's syndrome associated with a somatic frame shift mutation in the glucocorticoid receptor gene. J Clin Endocrinol Metab 1996; 81:124–129.

44 Hira Y, Sakai Y, Matsushima S: Effects of photoperiod and melatonin on the development of growth hormone cells and the pituitary-adrenal axis in the Djungarian hamster, *Phodopus sungorus*. Arch Histol Cytol 2001;64:211–222.

45 Filippa V, Mohamed F: ACTH cells of pituitary pars distalis of viscacha (lagostomus maximus maximus): immunohistochemical study in relation to season, sex, and growth. Gen Comp Endocrinol 2006;146:217–225.

46 Florio T: Somatostatin/somatostatin receptor signalling: phosphotyrosine phosphatases. Mol Cell Endocrinol 2008;286:40–48.

47 Srikant CB: Cell cycle dependent induction of apoptosis by somatostatin analog SMS 201-995 in AtT-20 mouse pituitary cells. Biochem Biophys Res Commun 1995;209:400–406.

48 Guillermet J, Saint-Laurent N, Rochaix P, Cuvillier O, Levade T, Schally AV, Pradayrol L, Buscail L, Susini C, Bousquet C: Somatostatin receptor subtype 2 sensitizes human pancreatic cancer cells to death ligand-induced apoptosis. Proc Natl Acad Sci USA 2003;100:155–160.

49 Gore AC, Attardi B, DeFranco DB: Glucocorticoid repression of the reproductive axis: effects on GNRH and gonadotropin subunit MRNA levels. Mol Cell Endocrinol 2006; 256:40–48.

50 Florio T, Arena S, Thellung S, Iuliano R, Corsaro A, Massa A, Pattarozzi A, Bajetto A, Trapasso F, Fusco A, Schettini G: The activation of the phosphotyrosine phosphatase eta (r-PTPε) is responsible for the somatostatin inhibition of PC Cl3 thyroid cell proliferation. Mol Endocrinol 2001;15:1838–1852.

51 Held-Feindt J, Forstreuter F, Pufe T, Mentlein R: Influence of the somatostatin receptor sst2 on growth factor signal cascades in human glioma cells. Brain Res Mol Brain Res 2001;87:12–21.

52 Hess RS, Saunders HM, Van Winkle TJ, Ward CR: Concurrent disorders in dogs with diabetes mellitus: 221 cases (1993–1998). J Am Vet Med Assoc 2000;217:1166–1173.