

Inhibitory effects of SOM230 on adrenocorticotrophic hormone production and corticotroph tumor cell proliferation *in vitro* and *in vivo*

(副腎皮質刺激ホルモン産生と腫瘍細胞増殖に対する *in vitro* 及び *in vivo* での SOM230 の阻害効果)

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ABSTRACT

Adrenocorticotrophic hormone (ACTH) production by pituitary corticotroph adenomas is the main cause of Cushing's disease. A drug that targets pituitary ACTH-secreting adenomas would aid treatment of Cushing's disease. Octreotide, a somatostatin receptor type 2 (SSTR2)-preferring somatostatin analogue, has no effect on ACTH secretion in patients with Cushing's disease. The multiligand SOM230 (pasireotide) displays a much higher affinity for SSTR1 and SSTR5 than octreotide and suppresses ACTH secretion in cultures of human corticotroph tumors to a greater extent than octreotide. In the present *in vitro* and *in vivo* study, we determined the effect of SOM230 on ACTH production and cell proliferation of AtT-20 corticotroph tumor cells. SOM230 decreased proopiomelanocortin (POMC) mRNA levels in AtT-20 cells and ACTH levels in the culture medium of these cells, suggesting that SOM230 suppresses ACTH synthesis and secretion in corticotroph tumor cells. SOM230 also decreased cell proliferation and both cyclic adenosine monophosphate response element-binding protein and Akt phosphorylation in AtT-20 cells. SSTR5 knockdown inhibited the SOM230-induced decreases in cell proliferation. Fluorescence-activated cell sorting analyses revealed that SOM230 did not attenuate cell cycle progression.

Tumor weight in mice xenografted with AtT-20 cells and treated with SOM230 was significantly lower than in AtT-20–xenografted control mice. SOM230 also significantly decreased plasma ACTH levels, and POMC and pituitary tumor transforming gene mRNA levels in the tumor cells. Thus, SOM230 inhibits ACTH production and corticotroph tumor cell proliferation *in vitro* and *in vivo*.

1. Introduction

Adrenocorticotrophic hormone (ACTH) is produced in pituitary corticotrophs when proopiomelanocortin (POMC) is cleaved to β -lipotropic hormone and ACTH by the prohormone convertase-1/3 processing enzyme (Benjannet et al., 1991; Fukuda et al., 2004). In addition, ACTH production may be coordinated with cell mass or growth in pituitary corticotroph tumor cells (Rhode and Gorski, 1991). Tumor growth is at least partially regulated by cell proliferation, cell cycle progression, and apoptosis. Pituitary tumor transforming gene (PTTG), a hallmark of pituitary tumors (Zhang et al., 1999; Chesnokova et al., 2011), facilitates cell cycle progression, increases pituitary cell proliferation, and promotes murine pituitary development (Chesnokova et al., 2012). Moreover, PTTG overexpression results in proliferation in pituitary gonadotrophs (Chesnokova et al., 2011). However, the relationship between ACTH production and cell proliferation in corticotroph tumor cells is poorly understood.

Cushing's disease is primarily caused by ACTH-producing pituitary adenomas (Nieman et al., 2008; Kageyama et al., 2013). Excision of the tumor from the pituitary is the primary treatment for Cushing's disease. However, if excision is unsuccessful, medical therapy is needed to treat the resultant hypercortisolism (Bertagna and Guignat, 2013). Some of the drugs used to treat this condition have shown potential

therapeutic benefits (Schteingart, 2009). Drugs that target pituitary ACTH-secreting adenomas include cyproheptadine, valproic acid, cabergoline, peroxisome proliferator-activated receptor-gamma agonists, and vasopressin antagonists, but these are less commonly used standard treatments for suppressing ACTH and cortisol production (Schteingart, 2009).

A more effective medical therapy is needed for the treatment of Cushing's disease. Various somatostatin receptor agonists have demonstrated inhibitory effects on tumor cell proliferation in pituitary tumors, including somatotroph and thyrotroph tumors. Octreotide is a somatostatin receptor type 2 (SSTR2)-preferring somatostatin analogue. However, it has no effect on ACTH secretion in patients with pituitary-dependent Cushing's disease. Novel somatostatin-dopamine chimeric molecules appear to be a promising approach (de Bruin et al., 2009; Ferone et al., 2009). The multiligand SOM230 (pasireotide) binds with high affinity to SSTR1, SSTR2, SSTR3, and SSTR5, and displays a 30- to 40-fold higher affinity for SSTR1 and SSTR5 than octreotide (Bruns et al., 2002). In human corticotroph adenomas, SSTR5 mRNA is expressed at much higher levels than other SSTR subtypes (Hofland et al., 2005). The SSTR subtype mRNAs expressed in AtT-20 cells are SSTR2, SSTR3 (low abundance), and SSTR5 (Hofland et al., 2005; Tateno et al., 2009).

SOM230 more potently suppresses ACTH secretion in cultures of human corticotroph tumors than octreotide (Hofland et al., 2005). SOM230 also inhibits ACTH release from the murine corticotroph tumor cell line AtT-20 (van der Hoek, 2005), and incubation with glucocorticoids does not affect the ability of SOM230 to suppress ACTH release in AtT-20 cells (Hofland et al., 2005). SOM230 suppresses ACTH secretion and POMC transcription, and ameliorates Cushing's symptoms in dogs (Castillo et al., 2011). In patients with Cushing's disease, SOM230 shows promising effects on decreasing the levels of plasma ACTH and urinary free cortisol (Feelders et al., 2010; Hofland, 2008). In addition to effects on ACTH secretion, the ability of a somatostatin analogue to suppress growth of the tumor cells would be important for a treatment of Cushing's disease. Indeed, SOM230 suppresses both cell proliferation and ACTH secretion in human pituitary corticotroph tumors (Batista et al., 2006). In an *in vivo* study in rats, SOM230 had a stronger inhibitory effect on ACTH and corticosterone secretion than octreotide (Silva et al., 2005). Nonetheless, the mechanisms through which the antiproliferative effects of SOM230 are exerted in corticotroph adenoma *in vivo* remain to be established.

In the present study, we confirmed the effects of SOM230 on ACTH production and newly explored the effects on cellular proliferation in AtT-20 corticotroph tumor

cells. To elucidate further the possible effects of SOM230 *in vivo*, we then examined for the first time the effects of SOM230 on various parameters, including body weight, tumor weight, tumor POMC and PTTG mRNA levels, and plasma ACTH and corticosterone levels, in AtT-20–xenografted mice.

2. Materials and Methods

2.1. Materials

SOM230 was kindly provided by Novartis Pharma AG (Basel, Switzerland).

2.2. Cell culture

AtT-20 pituitary corticotroph tumor cells were cultured in a T75 culture flask with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, and 100 U/mL penicillin at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The cells were plated in 6-well plates at 15.0×10^4 cells/cm² for 2 days before each experiment and the medium was changed every 48 h.

On day 3, to remove the effect of factors contained in FBS, the cells were washed and then starved overnight with DMEM supplemented with 0.2% bovine serum albumin prior to each experiment. At the end of each experiment, total cellular RNA or protein was collected and stored at –80°C until the relevant assay was performed.

2.3. RNA extraction

The cells were incubated with medium alone (control) or medium containing SOM230 for the indicated times. To examine the dose-dependent effects of SOM230, the

cells were incubated for the indicated times with medium alone (control) or medium containing increasing concentrations of SOM230 (1–100 nM). At the end of each experiment, total cellular RNA was extracted using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized from total RNA (0.5 µg) using random hexamers as primers with the SuperScript First-Strand Synthesis System for Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions.

2.4. Real-time RT-PCR

Total cellular RNA extraction and cDNA synthesis were performed as described previously (Kageyama et al., 2009; Kageyama et al., 2010). The resulting cDNA was then subjected to real-time PCR as follows. The expression levels of mouse POMC, SST2/5, and PTTG mRNA were evaluated using quantitative real-time PCR with specific sets of primers and probes (Assays-on-Demand Gene Expression Products; Applied Biosystems, Foster City, CA). β 2-microglobulin (B2MG) was used as a housekeeping gene to standardize expression levels because B2MG mRNA levels did not change during any treatments in this study. Each reaction consisted of 1 × TaqMan

Universal PCR Master Mix (Applied Biosystems), 1 × Assays-on-Demand Gene Expression Products (Mm00435874_m1 for mouse POMC, Mm00436685_g1 for mouse SST2, Mm01307775_s1 for mouse SST5, Mm00479224_m1 for mouse PTTG, and Mm00437762_m1 for mouse B2MG), and 1 µL cDNA in a total volume of 25 µL with the following parameters on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems): 95°C for 10 min and then 40 cycles at 95°C for 15 s and 60°C for 1 min.

The above assays involved specific sets of primers and a TaqMan probe spanning the exon–exon junction and should not, therefore, have been affected by DNA contamination. Data were collected and recorded with ABI PRISM 7000 SDS software (Applied Biosystems) and expressed as a function of the threshold cycle (CT). The amplification efficacies for each gene of interest and the housekeeping gene amplimers were found to be identical when analyzed with diluted samples.

2.5. Western blot analysis

Western blot analysis was performed to examine the protein levels of phosphorylated cyclic adenosine monophosphate response element-binding protein (pCREB)/CREB and phosphorylated Akt (pAkt)/Akt. The cells were washed twice with phosphate-buffered saline (PBS) and lysed with Laemmli sample buffer. Cell debris was

pelleted by centrifugation and the supernatant was recovered. The samples were boiled and subjected to electrophoresis on a 4%–20% gradient polyacrylamide gel, and the proteins were transferred to a polyvinylidene fluoride membrane (Daiichi Kagaku, Tokyo, Japan). After blocking with Detector Block® buffer (Kirkegaard & Perry Laboratories, Gaithersburg, MD), the membrane was incubated for 1 h with each antibody [anti-pCREB (1:500 dilution)/CREB (1:500 dilution) and anti-pAkt (1:500 dilution)/Akt (1:2000 dilution) antibodies, all from Cell Signaling Technology, Beverly, MA], washed with PBS containing 0.05% Tween 20, and incubated with horseradish peroxidase-labeled anti-rabbit immunoglobulin G (Daiichi Kagaku). The chemiluminescent substrate SuperSignal West Pico (Pierce Chemical Co., Rockford, IL) was used for detection and the membrane was exposed to BioMax film (Eastman Kodak Co., Rochester, NY).

2.6. ACTH assay

The cells were incubated at 37 °C for 24 h with the indicated concentrations of SOM230. The medium was then aspirated and the ACTH levels in the supernatants were measured using an ACTH enzyme-linked immunosorbent assay (ELISA) kit (MD Bioproducts, Zurich, Switzerland). Plasma ACTH levels in mice were also measured

using the kit. All samples from each experiment were determined in the same assay.

The intraassay and interassay coefficients of variation (CV) were 6.7% at 42.2 pg/ml and 7.1% at 42.3 pg/ml, respectively. Cross-reactivities with ACTH (1-24), ACTH (18-39), and α -melanocyte-stimulating hormone were -3.4%, -2.4%, and -1.7%, respectively.

2.7. Cell proliferation assay

The cells were incubated at 37 °C for 48 h with the indicated concentrations of SOM230. Viable cells were measured using a Cell Counting Kit-8 (Dojin, Kumamoto, Japan). All samples from each experiment were determined in the same assay.

2.8. Cell death detection assay

The cells were incubated at 37 °C for 24 h with the indicated concentrations of SOM230. DNA fragmentation was measured using a Cell Death Detection ELISA Kit (Roche, Penzberg, Germany), and each enrichment factor was calculated according to the manufacturer's instructions.

2.9. Cell cycle analysis

AtT-20 cells were incubated for 24 h with medium alone (control) or medium

containing 100 nM SOM230. The cells were collected by trypsinization, pelleted by centrifugation, and suspended in Triton X-100. The cells were treated at 37 °C for 30 min with 0.5% RNase A and stained with propidium iodide (50 µg/mL). Cellular DNA content was analyzed with fluorescence-activated cell sorting (FACS) analysis and the cell cycle profiles were determined with BD FACSDiva™ software (Becton Dickinson, Franklin Lakes, NJ).

2.10. RNA interference experiments

SSTR5 and control small interfering RNAs (siRNAs) were designed and purchased from QIAGEN. The cells were transfected with siRNA and HiPerFect transfection reagent (QIAGEN) according to the manufacturer's protocol. For a measurement of POMC and PTTG mRNA levels, the cells, seeded into 12-well plates at a density of 12×10^4 cells/well, were incubated for 24 h in 1 mL of culture medium containing siRNA for either control (siControl) or SSTR5 (siSSTR5, Mm_Sstr5_5), and then incubated with SOM230. The expression of POMC, PTTG, and B2MG mRNA was examined by quantitative RT-PCR. For a measurement of cell proliferation, the cells, seeded into 96-well plates at a density of 1.5×10^4 cells/well, were incubated for 24 h in 200 µL of culture medium containing siRNA for either control (siControl) or SSTR5

(siSSTR5). The cells were incubated with vehicle or 100 nM SOM230 for 48 h. Viable cells were measured using a Cell Counting Kit-8.

2.11. Xenograft experiments

All animal studies were approved by the Institute for Animal Experiments, Hirosaki University School of Medicine, and Institutional Review Board of Hirosaki University School of Medicine. Male KSN/Slc nude mice at 8 weeks of age (approximate 22–24 g) were purchased from Japan SLC Inc. (Shizuoka, Japan) and maintained in pathogen-free conditions. Mice were subcutaneously injected with AtT-20 cells (5×10^6 /mouse) in vehicle (200 μ l) and divided randomly into two groups: control (vehicle only) and SOM230 (1.5 μ g/mouse per day). SOM230, dissolved in vehicle (ethanol and 0.9% saline), was administered via Alzet osmotic minipumps (DURECT Corporation, Cupertino, CA) for 14 days from 1 week after AtT-20 cell injection. 1.5 μ g/mouse per day of SOM230 was chosen, because it corresponds to 50 μ g/kg BW, and this dose has been shown to produce enough long-lasting effects on pituitary hormone secretion in rodents (Imhof et al., 2011). At the end of the experiment, the mice were killed and tumor weights were measured after their careful resection. All mice were killed from 2:00 to 3:00 PM. The tumors were used for a quantitative real-time RT-PCR assay. Trunk blood

was also collected for blood glucose and corticosterone assays. Blood glucose levels were measured using Nipro StatStrip XP (Nipro, Osaka, Japan) at the time of the experiment.

2.12. Corticosterone assay

Blood was centrifuged and a rodent corticosterone ELISA kit (Endocrine Technologies, Newark, CA) was used to measure plasma corticosterone levels. All samples from each experiment were determined in the same assay.

2.13. Statistical analysis

Each *in vitro* experiment was performed at least three times. Samples were provided in triplicate for each group of experiments. Each value is expressed as the mean \pm standard error of the mean. For *in vitro* studies, statistical analysis was performed with analysis of variance (ANOVA), followed by a Fisher's protected least-significant difference post hoc test. For *in vivo* studies, statistical analysis was performed with unpaired Student's t-test. The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Effects of SOM230 on ACTH and POMC, SSTR2/5, and PTTG mRNA levels

AtT-20 cells were incubated with SOM230 to determine its effects on the time- and dose-dependent changes in POMC mRNA levels. A time course study showed that 100 nM SOM230 significantly decreased POMC mRNA levels (ANOVA; $P < 0.05$, Fig. 1A) with POMC mRNA levels falling to 72% of the control value within 6 h of the addition of 100 nM SOM230 (Fig. 1A). POMC mRNA levels decreased in a dose-dependent manner (ANOVA; $P < 0.05$), with significant effects observed from 10 to 100 nM (Fig. 1A). A time course study showed that 100 nM SOM230 significantly decreased ACTH levels within 24 h of the addition of 100 nM SOM230 (ANOVA; $P < 0.05$, Fig. 1B). ACTH levels in the medium also decreased in a dose-dependent manner (ANOVA; $P < 0.001$), with significant effects observed from 1 to 100 nM SOM230 (Fig. 1B). SSTR2/5 mRNA levels were unaltered within 24 h of the addition of 100 nM SOM230 (Fig. 1C). PTTG mRNA levels fell to 82% of the control value within 24 h of the addition of 100 nM SOM230 (Fig. 1D). PTTG mRNA levels decreased in a dose-dependent manner (ANOVA; $P < 0.05$), with significant effects observed at 100 nM (Fig. 1D).

3.2. Time-dependent changes in SOM230-induced CREB and Akt phosphorylation

AtT-20 cells were incubated with 100 nM SOM230 to determine its effects on CREB and Akt phosphorylation. A time course study showed that SOM230 decreased CREB phosphorylation from 5 min to 2 h and Akt phosphorylation from 30 min to 6 h in AtT-20 cells (Fig. 2).

3.3. Effects of SOM230 on cell proliferation and cell death

AtT-20 cells were incubated with SOM230 to determine its effects on the dose-dependent changes in cell proliferation and cell death. Cell proliferation decreased in a dose-dependent manner (ANOVA; $P < 0.05$), with significant effects observed from 10 to 100 nM (Fig. 3A). To examine whether SOM230 induced apoptosis, cytoplasmic histone-associated DNA fragmentation was determined. There was a nonsignificant increase in DNA fragmentation upon the addition of 100 nM SOM230 (ANOVA; $P = 0.38$; Fig. 3B).

3.4. Effects of SSTR5 on POMC and PTTG mRNA levels, and cell proliferation

(Fig. 4)

We then examined the functional role of SSTR5 in AtT-20 cells, since SSTR5 might

be involved in the regulation of POMC and PTTG mRNA levels, and cell proliferation. SSTR5 mRNA levels were reduced by 35% in cells transfected with siRNA against the SSTR5 gene. SSTR5 knockdown did not have any significant changes in basal POMC and PTTG mRNA levels compared with the control (Figs. 4A and 4B). POMC and PTTG mRNA levels were decreased by incubation with SOM230 (Figs. 4A and 4B). SSTR5 knockdown failed to inhibit the SOM230-induced decreases in POMC mRNA levels (Fig. 4A), while it significantly inhibited the SOM230-induced decreases in PTTG mRNA levels (Fig. 4B). SSTR5 knockdown did not have any significant effect on basal cell proliferation compared with the control (Fig. 4C). Cell proliferation was decreased by incubation with SOM230 (Fig. 4C). SSTR5 knockdown significantly inhibited the SOM230-induced decreases in cell proliferation (Fig. 4C).

3.5. Effects of SOM230 on cell cycle profiles

Cell cycle distribution was assessed using flow cytometry. FACS analyses revealed that the percentages of cells in both G0/G1 and G2/M phases were unaltered after incubation with 100 nM SOM230 (Fig. 5A and B).

3.6. Effects of SOM230 on parameters in AtT-20-xenografted mice

AtT-20–xenografted mice produce high levels of plasma corticosterone levels, and have been used as an animal model of ACTH-dependent Cushing’s disease (Taguchi et al., 2006; Fukuoka et al., 2011). AtT-20–xenografted mice were treated with SOM230 to determine its effects on corticotroph tumor growth and function *in vivo*. SOM230, dissolved in vehicle, was administered via osmotic minipumps for 14 days from 1 week after AtT-20 cell injection (Fig. 6A). In control (vehicle) mice xenografted with AtT-20 cells, body weight was decreased during the study period (14 days). The decrease in body weight was smaller, although not significantly, following treatment with SOM230 (Fig. 6B). Tumor weight in mice treated with SOM230 was significantly lower than that in control mice ($P < 0.05$; Fig. 6C). SOM230 also significantly decreased tumor POMC and PTTG mRNA levels compared with control ($P < 0.05$; Fig. 6D and 6E). Finally, SOM230 significantly decreased plasma ACTH levels compared with control ($P < 0.005$; Fig. 6F). There is a trend toward a decrease in plasma corticosterone levels ($P = 0.10$), following treatment with SOM230 (Fig. 6G). Blood glucose levels were unaltered in SOM230-treated mice (Fig. 6H).

4. Discussion

Recent studies suggested that SOM230 is a promising drug for treating ACTH-dependent Cushing's disease. In human corticotroph adenomas, SSTR5 mRNA is expressed at much higher levels than other SSTR subtypes (Hofland et al., 2005). Octreotide shows inhibitory effects on corticotrophin-releasing factor (CRF)-induced, but not basal, ACTH secretion in human corticotroph tumor cells, whereas SOM230 shows inhibitory effects on basal ACTH secretion in these cells (Hofland et al., 2005). In this study, we found that SOM230 decreased POMC mRNA levels in AtT-20 cells and the basal levels of ACTH in the culture medium of these cells. These results suggest that SOM230 suppresses autonomic production, as well as the synthesis and release, of ACTH in corticotroph tumor cells.

SOM230 decreased POMC mRNA levels within 6 h, while it significantly did ACTH levels in the medium within 24 h, but not 2 h and 6 h. The difference in kinetics between POMC mRNA and ACTH levels was found in this study. The expression levels of mRNA are determined mainly by both transcription, such as a synthesis of mRNA, and post-transcriptional processes. On the other hand, ACTH levels in the medium would depend on various factors, such as ACTH synthesis, ACTH secretion, and cell proliferation. Thus, SOM230-induced decreases in total production or accumulation of

ACTH within 24 h would cause the significant changes in the study.

We previously confirmed that CREB phosphorylation was increased by CRF via the protein kinase A (PKA) pathway in AtT-20 cells (Kageyama et al., 2007). The PKA pathway has an important role in the regulation of POMC gene and the desensitization of CRF receptor type 1 by CRF in corticotroph cells (Kageyama et al., 2006; Kageyama et al., 2007). In AtT-20 cells, the PKA pathway participates in gene regulation via CREB phosphorylation. Endogenous SSTR2, SSTR3, and SSTR5 signals constitutively restrain cyclic AMP-PKA and mitogen-activated protein kinase pathways, leading to suppression of ACTH production (Tsukamoto et al., 2010). Our results demonstrated that SOM230 decreased CREB phosphorylation, resulting in inhibited synthesis and release of ACTH. On the other hand, treatment of AtT-20 cells with SOM230 for 24 h failed to sustain the reduction of POMC mRNA obtained at 2 h and 6 h.

SOM230-induced decreases in POMC gene transcription may be rapidly recovered. For example, SSTR3 is unique in that it was rapidly downregulated during prolonged agonist exposure (Lesche et al., 2009). Due to its rapid downregulation, the SSTR3 seems to be a less favorable target for long-term administration of somatostatin analogs (Lesche et al., 2009). Further study in the future is expected at this point.

SOM230 effects are mediated via SSRTs, such as SSTR1, SSTR2, SSTR3, and

SSTR5. Glucocorticoids often affect receptor sensitivity or number. However, pretreatment with glucocorticoids fails to influence cell sensitivity to the inhibitory effect of SOM230 (Hofland et al., 2005). Given that SSTR5 is more resistance to glucocorticoids, the effects of SOM230 may last longer via these receptors. Agonists also modulate the expression levels or sensitivity of the receptors (Moriyama et al., 2005).

Among SSTRs, SSTR3 is rapidly downregulated by agonists. In fact, SOM230 stimulated a rapid downregulation of SSTR3, but not SSTR2 or SSTR5 (Lesche et al., 2009). In the present study, SSTR2 and SSTR5 mRNA levels were not changed by SOM230, suggesting that both receptors might not be downregulated by the agonists.

SOM230 gradually decreased PTTG mRNA levels in AtT-20 cells. The difference in kinetics between POMC and PTTG mRNA levels may be caused by SSTRs involved in the regulation of each gene. In fact, SSTR5 knockdown inhibited the SOM230-induced decreases in PTTG, but not POMC, mRNA levels. Therefore, the SOM230 effects on regulation of PTTG mRNA are mediated mainly via SSTR5, while SSTRs other than SSTR5 might contribute to the regulation of POMC mRNA. The differences of roles in SSTRs may contribute to the difference in kinetics between POMC and PTTG mRNA levels.

Generally, PTTG would be involved in cell proliferation. However, the

proliferation was suppressed at 10 nM SOM230, while PTTG mRNA was unaffected at this concentration in AtT-20 cells. Although PTTG is also known to facilitate cell cycle progression, SOM230 failed to modulate cell cycle profile in this study. In fact, effects of SOM230 on PTTG mRNA levels were not so strong in the *in vitro* study. These results, therefore, may suggest that PTTG is not involved in the reduction of the pituitary cell proliferation induced by SOM230 in this study.

Decreases in cell numbers may contribute to decreases in ACTH production. SOM230 decreased cell proliferation but did not significantly increase DNA fragmentation in AtT-20 cells. The data suggest that SOM230 does not induce apoptosis. FACS analyses also revealed that SOM230 did not modulate the percentages of G0/G1 and G2/M phase cells in AtT-20 cells. SOM230 may not attenuate cell cycle progression or cause cell arrest, resulting in the proliferation of AtT-20 cells. The mechanisms through which SOM230 decreased cell proliferation in AtT-20 cells are still unclear. However, SSTR5 knockdown inhibited the SOM230-induced decreases in cell proliferation. The results suggest that the SOM230 effects on cell proliferation are mediated at least via SSTR5.

SOM230 decreased Akt phosphorylation. The phosphoinositide 3-kinase/Akt pathway is an intracellular signaling pathway that contributes to apoptosis or

antiproliferative action in pituitary tumor cells (Theodoropoulou et al., 2006; Ferone et al., 2009). This pathway is overactive in a variety of cancer cells, thereby reducing apoptosis and allowing proliferation. In fact, Akt expression levels are upregulated in pituitary tumors, including corticotroph tumor cells, compared with normal pituitary cells (Muşat et al., 2004; Yang et al., in press). Therefore, decreases in cell proliferation may be mediated by the Akt pathway in pituitary corticotroph tumor cells.

In control mice xenografted with AtT-20 cells, body weight was decreased. The present results are consistent with those of earlier studies, which show that mice with Cushing's syndrome lose weight (Taguchi et al., 2006). Treatment with SOM230 decreased tumor POMC, suggesting that SOM230 also suppresses ACTH synthesis *in vivo*. Tumor weight in mice treated with SOM230 was lower than that in control mice, and SOM230 may suppress cell proliferation *in vivo*. In this study, SOM230 decreased PTTG mRNA levels in AtT-20 cells. SOM230 more potently decreased tumor PTTG mRNA levels *in vivo* than *in vitro*. Together, these findings indicate that PTTG may be partially involved in SOM230 suppression of cell proliferation in mice xenografted with AtT-20 cells. Continuous injection of SOM230 *in vivo* may affect corticotroph suppression of cell proliferation. The high affinity of SOM230 for SSTR5 may also inhibit insulin secretion from the pancreas and increase blood glucose levels (Schmid

and Brueggen, 2012; Henry et al., 2013). However, blood glucose levels in this study were unaltered in SOM230-treated mice. Cushing's disease is characterized by ACTH-dependent excessive circulating glucocorticoid concentrations. Therefore, plasma ACTH and corticosterone levels were measured in *in vivo* study (Taguchi et al., 2006; Fukuoka et al., 2011). Finally, plasma ACTH levels were significantly decreased, while corticosterone levels were not significantly changed, following treatment with SOM230. SOM230 suppressed plasma ACTH levels directly via the tumor production even *in vivo*. On the other hand, plasma corticosterone levels may be modulated via other stress factors or conditions in addition to stimulation of ACTH *in vivo*. In patients with Cushing's disease, late night salivary cortisol is reported to be a useful biomarker to assess the early response to SOM230 (Trementino et al., 2014). Additionally, the dose, 50 µg/kg BW, of SOM230 used in this study, corresponds to almost 3 mg/day in human. A maximum dose of 1.8 mg/day has been used subcutaneously in patients with Cushing's disease (Calao et al., 2012). Further studies, including those with increased doses of SOM230 or other means of administration, may confirm these results.

In conclusion, the present study demonstrated that SOM230 decreases POMC mRNA levels in AtT-20 cells and ACTH levels in the culture medium of these cells. SOM230 decreases cell proliferation in AtT-20 cells. Tumor weight in mice xenografted with

AtT-20 cells and treated with SOM230 is significantly lower than that of control mice.

SOM230 also significantly decreases plasma ACTH levels, and tumor POMC and PTTG mRNA levels. Thus, SOM230 shows inhibitory effects on ACTH production and corticotroph tumor cell proliferation *in vitro* and *in vivo*.