Inhibition of Hormone Secretion in GH-Secreting Pituitary Adenomas by Receptor-Subtype Specific Somatostatin Analogues *in vitro*

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Abstract. The aim of the study was to determine the inhibitory effects of somatostatin analogues with relative specificity to somatostatin receptor subtype 2 (SSTR2) (BIM-23197), subtype 5 (SSTR5) (BIM-23268), and their combination on GH and PRL secretion in acromegalic adenomas *in vitro*.

Three types of answer were observed: 1. In one resistant adenoma no inhibition was achieved. 2. The GH secretion in six adenomas was suppressed significantly more ($p < 0.01$ or $p < 0.001$ using Man-Whitney U-test in concentration range of 10^{-12} to 10^{-8} mol/l) with SSTR2 specific analogue BIM-23197 with no additive effect of compounds combination. 3. In three adenomas the potency of BIM-23197 and BIM-23268 was almost equal and the combination of these SSTR2 and SSTR5 specific compounds had statistically significant additive effect ($p < 0.05$ or $p < 0.01$ in concentration range of 10^{-12} to 10^{-8} mol/l). PRL secretion of five adenomas was more suppressed with SSTR5 specific BIM-23268 (statistically significant in concentrations 10^{-10} to 10^{-8} mol/l).

In conclusion the somatostatin analogue BIM-23268 had an additive effect on suppression of GH secretion in a subset of adenomas, where both SSTR2 and SSTR5 were involved. This effect was not observed in the majority of tumours, where the inhibitory effect seems to be mediated *via* SSTR2 only.

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Introduction

Acromegaly is a debilitating disorder that usually develops over many years and in the vast majority of cases is caused by long-term hypersecretion of growth hormone (GH) from a benign pituitary adenoma (Melmed 1990). Surgery remains the method of choice in most cases (Melmed et al. 1998) but only approximately 70–80 % of patients with microadenomas and less than 50 % of patients with macroadenomas achieve sufficient control of disease activity (Ross and Wilson 1988; Melmed et al. 1998). As it usually takes many years since acceptable decrease of GH hypersecretion is achieved after irradiation, somatostatin analogues are widely used for acromegaly treatment both after operation and/or irradiation (Lamberts 1988; Freda 2002) and as a primary therapy (Newman et al. 1998).

Somatostatin analogues and native somatostatin elicit their biological effects by activating membrane G protein-coupled somatostatin receptors (SSTR). There are five distinct somatostatin receptors, types SSTR1–SSTR5 (Patel 1999; Schonbrunn 1999). The receptors differ in their regulatory/signalling pathways, and the affinity to which the somatostatin analogues bind to them (Reisine and Bell 1995). The two analogues of somatostatin available for clinical use are the cyclic octapeptides octreotide and lanreotide. A significant number of GH-secreting pituitary tumours are resistant to octreotide and lanreotide, and this may be explained in part by variable tumoral expression or reduced receptor density of subtypes 2 or 5 on these tumours (Reubi and Landolt 1989; Jaquet et al. 2000). Studies using subtype-selective somatostatin analogues demonstrated the involvement of both SSTR2 and SSTR5 receptor subtypes in regulating GH secretion from human pituitary adenomas (Shimon et al. 1997b; Jaquet et al. 2000). As octreotide and lanreotide both have 12- to 18-fold lower binding affinities for SSTR5 than for SSTR2 (Shimon et al. 1997a) it has been hypothesised, that their partial efficacy to control GH hypersecretion in some acromegalic patients could be the consequence of their lower affinity for the SSTR5 subtype (Saveanu et al. 2001).

In the present study we investigated the potency of somatostatin analogues with preferential affinity to SSTR2 (BIM-23197), to SSTR5 (BIM-23268) and their combination to inhibit GH and prolactin (PRL) secretion from GH-secreting pituitary adenoma cells cultivated *in vitro*.

Materials and Methods

Patients

The study has been approved by the Ethics Committee of the Charles University in Prague, Faculty of Medicine in Hradec Králové and the patients signed informed consent. Ten acromegalic patients (six women and four men), aged 24–74 years,

presenting with macroadenoma were studied. Their endocrine status and neuroradiological characterisation of the pituitary adenomas were documented before treatment. Basal GH levels were the mean of three random samples obtained. The basal IGF-I value was evaluated under fasting conditions between 8 and 10 o'clock. All patients underwent transsphenoidal surgery. The clinical endocrine and tumoral status of each patient is summarised in Table 1. With the exception of one patient pre-treated with octreotide, no specific treatment of acromegaly was introduced before surgery. Histological and immunocytochemical characteristics of adenomas are summarised in Table 2.

Table 1. Clinical endocrine and tumoral status evaluated by MRI in acromegalic patients before surgery

Case No.	Age (years)	Sex	GH (μg/l)	PRL (μg/l)	IGF-I (μg/l)	Adenoma size (mm)	Extrasellar extension*
1	62	F	11.7	8.4	1429	$12 \times 12 \times 12$	infra
2	61	F	7.2	9.9	1426	20×15	supra
3	24	M	191	14.1	955	$50 \times 40 \times 30$	supra, para
4	74	F	34	7.6	1400	9×11	no
5	45	M	67	28.7	1190	38×35	supra
6	73	M	>800	32.0	803	gigantic	supra, para, infra
7	46	F	166	38.8	945	30×24	supra, para, infra
8	33	F	42	103.0	1314	$12 \times 10 \times 15$	slightly supra
9	59	M	17	7.6	1342	$30 \times 18 \times 28$	supra, para
10	26	F	62	24.8	1672	30×28	supra, para

* infra, supra or parasellar tumour extension.

Hormone assays

GH was measured using commercial RIA kit (CIS Bio International, France) and PRL using chemiluminescent commercial assay (Bayer, Strawberry Hill, UK). After an ethanol-acid extraction, the plasma IGF-I assay was performed using the IGF-I RIA kit from Immunotech (Prague, Czech Republic).

Cell culture studies

Whole tumour tissue obtained at surgery with the exception of randomly chosen (at least two) parts for histological investigation was processed for cell culture studies. Cells were dissociated mechanically. After washing in serum-free D-MEM (Sigma, Steinheim, Germany) the number of viable cells was established by trypan blue exclusion method. Depending on the specimen size and consistency $3 - 45 \times 10^6$ isolated cells were obtained. Tumour cells were cultivated in D-MEM (Sigma) supplemented with 10% fetal calf serum. The cells were plated in multiwell culture

Table 2. Histological characteristics of adenomas

Case No.	Standard pathology	Immunocytochemistry GH	PRL	other hormones
1	chromophobic	+	–	n.p.
2	A++	++	–	n.p.
3	A+	+	–	–
4	A++	++	+	alpha
5	A+++	+	–	alpha
6	A+	++	+	–
7	chromophobic	++	+	–
8	A+++	+	++	alpha, FSH, TSH
9	A++	+	–	–
10	chromophobic	+	+	–

A+ to A+++, various degrees of acidophilia; n.p., not performed; alpha, alpha subunit of glycoprotein hormones.

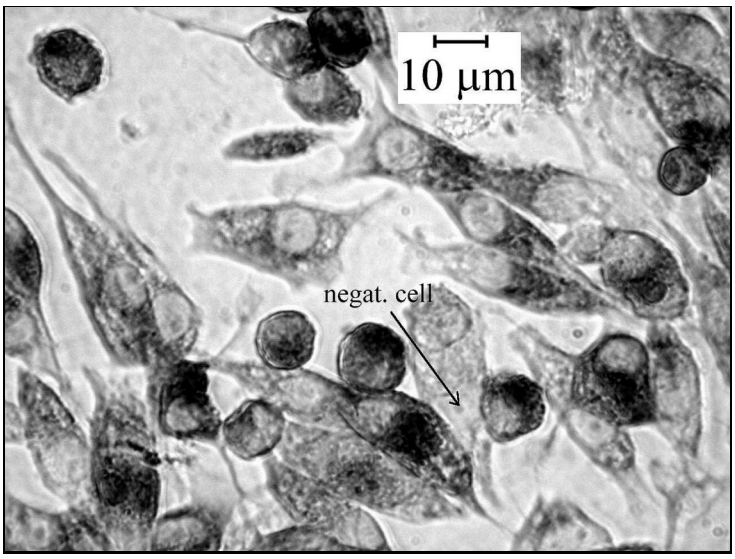

Figure 1. Adenoma cells after 10 days in culture. Immunocytochemical detection of growth hormone (GH) (streptavidin-biotin method with peroxidase and DAB, original magnificaton ×400 in inverted microscope). GH-positive cells stain brown (dark on this photomicrograph), arrow shows an example of a negative cell.

dishes coated with poly-L-lysine (Sigma) at a density of 3×10^4 cells/well. A representative culture is shown in Figure 1. After 3–5 days the medium was removed and replaced with serum-free D-MEM supplemented with studied somatostatin ana-

logues (and their combination) in a concentration range of 10^{-8} to 10^{-12} mol/l. At least four control wells with serum-free medium only, without any somatostatin analogue, were left on each multiwell plate. The concentration of GH and PRL was determined in each well before and after six hours' incubation in 5% CO_2 atmosphere at 37℃. The increase in concentration was calculated as a measure of the amount of hormone secreted. Each drug concentration was tested in duplicate to quadruplicate, depending on the quantity of cells obtained.

Compounds

The BIM compounds were provided by Biomeasure, Inc. (Milford, MA, USA). Two analogues with different receptor specificities were chosen: BIM-23197 (analogue A in further text) and BIM-23268 (analogue B in further text). Their binding characteristics to human SSTR subtypes were determined by radioligand receptor binding assays by Shimon et al. (1997a). The analogue A is relatively SSTR2 specific (IC_{50} was eighty times lower than that for analogue B) while analogue B binds preferentially to SSTR5 (IC_{50} was twenty six times lower than that for analogue A). The analogues were dissolved in 0.01 mol/l acetic acid containing 0.1% purified serum albumin. The drugs were stored at −20℃ as 10^{-5} mol/l solutions. For each experiment, fresh working solutions were prepared from a new aliquot.

Statistics

The secretion of GH and PRL in the presence of individual concentrations of somatostatin analogues and their combination was expressed in percent of the secretion in control wells, containing serum-free D-MEM only, which was set 100%. Besides, in each individual adenoma culture the EC_{50} value for the two somatostatin analogues and their combination has been estimated. The results were expressed as mean ± S.D. and the statistical significance were determined by the Mann-Whitney Rank Sum test. Sigma-Stat software (Jandel Scientific Corporation, San Rafael, CA, USA) was used.

Results

GH suppression

In one adenoma (No. 3) no suppression of GH secretion was observed not only with somatostatin analogues, but also with cyclic somatostatin-14 (data not shown) up to the concentration of 10^{-8} mol/l. This case was considered to be resistant to somatostatin and was excluded from further analysis. Mean suppression of GH secretion in the other nine cases is shown in Figure 2. The analogue B, preferentially binding SSTR5 inhibited GH secretion significantly less than analogue A with the preference to SSTR2 ($p < 0.01$ or $p < 0.001$ in the concentration range of 10^{-12} to 10^{-8} mol/l). The mean EC_{50} values are summarised in Table 3. It was 0.06 ± 0.04 nmol/l for analogue A and 1.9 ± 1.0 nmol/l for analogue B, respectively.

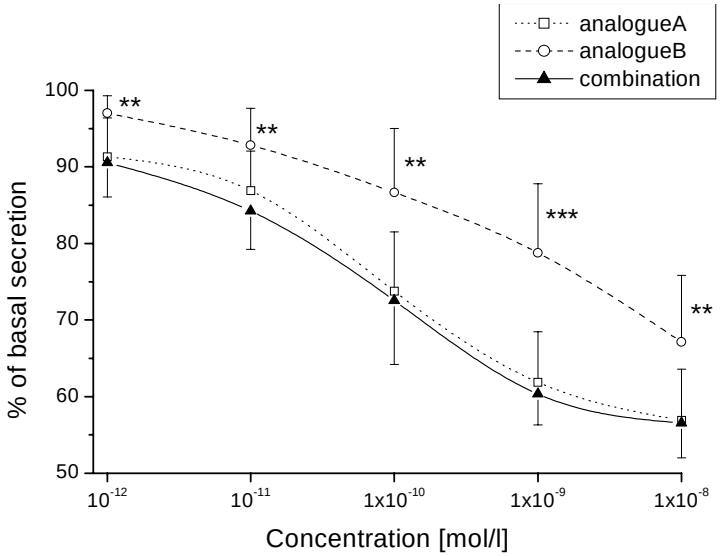

Figure 2. Suppression of growth hormone secretion from nine GH-secreting pituitary adenomas. The results are expressed for each concentration as mean ± S.D. in percent of secretion in control wells, containing vehicle only. Analogue A, BIM-23197; analogue B, BIM-23268; combination, their equimolar combination; $^{**}p < 0.01$; $^{***}p < 0.001$ for significance of difference in Man-Whitney Rank Sum test.

Table 3. EC_{50}values (mean ± S.D.) in nmol/l

Adenomas	Analogue A	Analogue B	A + B
GH secretion			
All adenomas	0.06 ± 0.04	1.9 ± 1.0*	0.05 ± 0.04
Adenomas No. 1, 4, 5, 8, 9,10	0.05 ± 0.02	2.5 ± 0.7**	0.06 ± 0.03
Adenomas No. 2, 6, 7	0.08 ± 0.03	0.07 ± 0.03	0.02 ± 0.01*
PRL secretion			
Adenomas No. 4, 6, 7, 8, 10	1.4 ± 0.9**	0.12 ± 0.06	0.09 ± 0.04

Analogue A, BIM-23197; analogue B, BIM-23268; A + B, their equimolar combination; * $p < 0.05$; ** $p < 0.01$ for significance of difference in Man-Whitney Rank Sun test.

The difference is statistically significant ($p = 0.02$). The combination of these two compounds was not more effective than the analogue A alone.

However, in three cases (No. 2, 6, 7) the SSTR5 binding analogue B was almost equally as effective as the analogue A. In these three cases the combination of the analogues was at corresponding concentrations significantly more potent than each

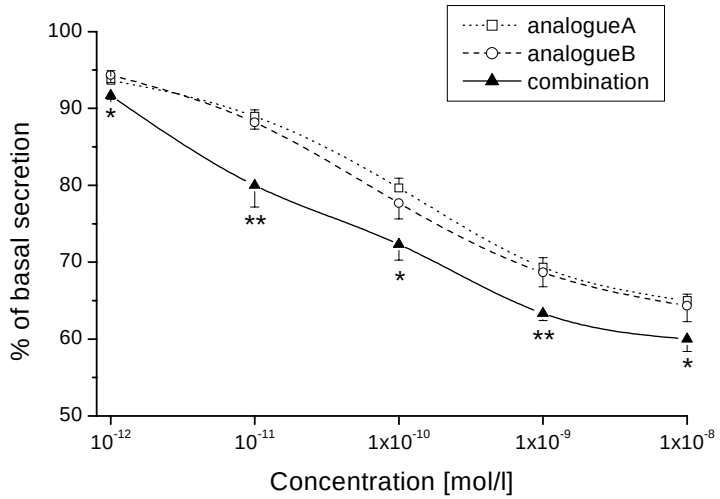

Figure 3. Suppression of growth hormone secretion in three GH-secreting pituitary adenomas, where analogues A and B were equally potent. The results are expressed for each concentration as mean ± S.D. in percent of secretion in control wells, containing vehicle only. Analogue A, BIM-23197; analogue B, BIM-23268; combination, their equimolar combination; * $p < 0.05$; ** $p < 0.01$ for significance of difference in Man-Whitney Rank Sum test.

of the compounds alone ($p < 0.05$ or $p < 0.01$ for % of basal hormone secretion in concentration range of 10^{-12} to 10^{-8} mol/l, Figure 3). The mean EC_{50} value for combination was 0.02 ± 0.01 nmol/l, significantly lower than for individual compounds ($p = 0.05$, Table 3). In the remaining 6 adenomas the greater effect of SSTR2 binding analogue A was even more evident than in the whole group ($p < 0.01$ or $p < 0.001$ for % of basal hormone secretion in concentration range of 10^{-12} to 10^{-8} mol/l, Figure 4). The mean EC_{50}value for analogue A was 0.05 ± 0.02 and for analogue B 2.5 ± 0.01 nmol/l, respectively ($p < 0.01$, Table 3).

Prolactin suppression

In five adenoma cell cultures (No. 4, 6, 7, 8, 10) PRL secretion was sufficient to allow secretion study. The results are shown in Figure 5. The analogue B with the preference to SSTR5 inhibited PRL secretion significantly more than analogue A. The difference in % of basal hormone secretion was statistically significant in the concentration range of 10^{-10} to 10^{-8} mol/l ($p < 0.05$ or $p < 0.01$). The combination of both analogues did not have any additive effect. The mean EC_{50} value for analogue A was 1.4 ± 0.9 nmol/l, significantly higher ($p < 0.01$) than that for analogue B (0.12 ± 0.06 nmol/l) and analogues combination (0.09 ± 0.04), respectively.

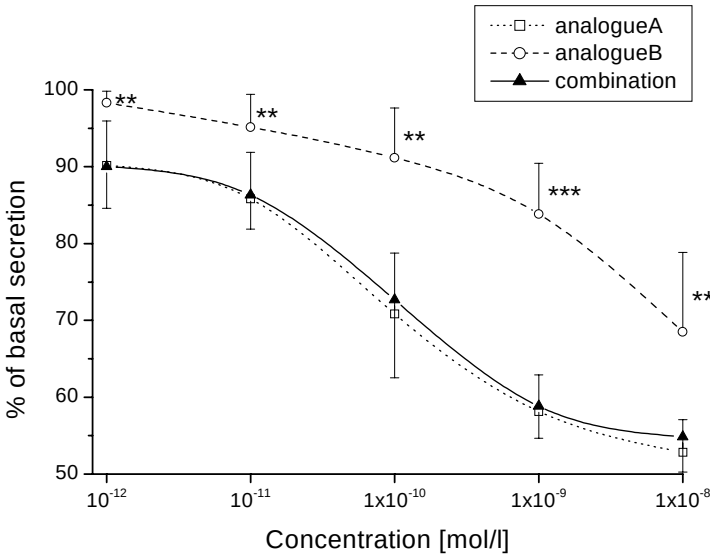

Figure 4. Suppression of growth hormone secretion in six GH-secreting pituitary adenomas, where analogue A was more potent than analogue B. The results are expressed for each concentration as mean ± S.D. in percent of secretion in control wells, containing vehicle only. Analogue A, BIM-23197; analogue B, BIM-23268; combination, their equimolar combination; ** $p < 0.01$; *** $p < 0.001$ for significance of difference in Man-Whitney Rank Sum test.

Discussion

Since their introduction to the acromegaly treatment (Lamberts et al. 1985) it has been clear that the therapeutic response to somatostatin analogues is individually variable. Normalisation of IGF-I level can be achieved in only about one half of the acromegalics and in some patients the GH levels do not decrease at all (Marek et al. 1994; Freda 2002). A mutation of SSTR has been recently described in a case of resistant adenoma (Ballare et al. 2001), but this is probably a rare occasion. The cause of the poor response of some adenomas to treatment with somatostatin analogues seems to be variable expression of somatostatin receptors. The correlation between sensitivity to somatotstatin and SSTR density has been shown by receptor autoradiography (Reubi and Landolt 1989) and reverse transcriptase PCR (RT-PCR) analysis (Jaquet et al. 2000).

After discovery of five SSTR subtypes, their tissue distribution and role in GH and PRL regulation has been extensively studied. In primary human foetal pituitary cultures it has been demonstrated that both SSTR2 and SSTR5 are involved in GH and thyroid-stimulating hormone regulation, while PRL secretion are suppressed only by SSTR2-specific compounds (Shimon et al. 1997a). Numerous qualitative studies, using RT-PCR, ribonuclease protection assay, or *in situ* hy-

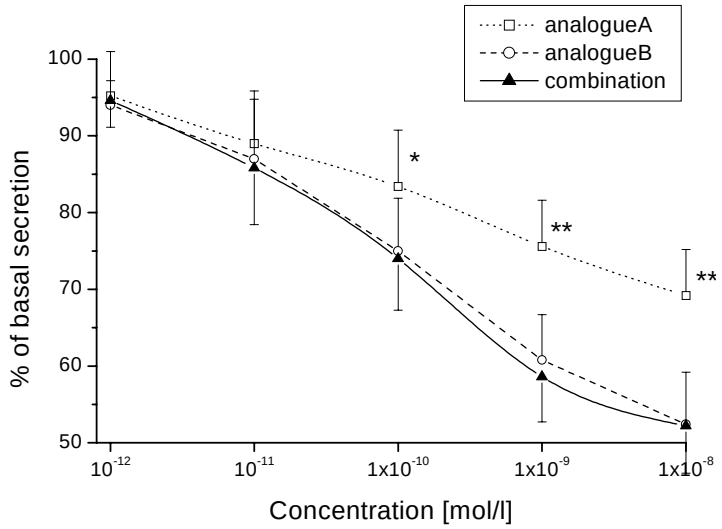

Figure 5. Suppression of prolactin secretion in five mixed GH- and PRL-secreting pituitary adenomas. The results are expressed for each concentration as mean ± S.D. in percent of secretion in control wells, containing vehicle only. Analogue A, BIM-23197; analogue B, BIM-23268; combination, their equimolar combination; * $p < 0.05$; ** $p < 0.01$ for significance of difference in Man-Whitney Rank Sum test.

bridisation techniques have been performed in human pituitary GH-secreting adenomas (Greenman and Melmed 1994a,b; Miller et al. 1995; Panetta and Patel 1995; Murabe et al. 1996; Nielsen et al. 1998). SSTR2 transcript was identified almost always and SSTR5 in about three quarters of all adenomas; a highly variable expression of SSTR1 and SSTR3 was found and the SSTR4 transcript has never been observed. Recently French group have found both SSTR2 and SSTR5 mRNA in all acromegalic adenomas studied using quantitative RT-PCR, with SSTR5 mRNA being quantitatively more abundant (Jaquet et al. 2000; Saveanu et al. 2001). Only SSTR2 mRNA quantity correlated with sensitivity to native somatostatin *in vitro* (Jaquet et al. 2000).

In our series, considering the whole group of adenomas studied, the suppression of GH secretion was significantly higher with somatostatin analogue A preferentially binding SSTR2 than with SSTR5 specific analogue B through the whole concentration range used (Fig. 1). This reflects the dominant role of SSTR2 in GH regulation in most acromegalic adenomas. No significant additive effect of combination with the analogue B could be detected. However, in a subgroup of three adenomas studied, the analogue B preferentially binding SSTR5 was equally as effective as the SSTR2 specific analogue A (Fig. 3). In these tumours both receptor subtypes seem to be equally involved in the regulation of GH secretion.

This finding is in keeping with results of Jaquet and co-workers (Jaquet et al. 2000), who postulated that heterogeneous SSTR subtype functionality might reflect adenoma origin from cells at different stage of somatolactotroph development.

On the contrary to the adenomas with the SSTR2 predominant regulation in these three tumours we were able to demonstrate an additive effect of the combination of analogue with preference to SSTR2 (A) with the analogue B, preferentially binding SSTR5. While such an additive effect has been described by Shimon and co-workers in one adenoma studied (Shimon et al. 1997b), in another series a lack of synergism between both compounds at maximally effective concentrations was observed (Jaquet et al. 2000). In a recent study (Saveanu et al. 2001) the bispecific somatostatin analogue BIM-23244, binding with high affinity to both SSTR2 and SSTR5, has been shown to be more potent than octreotide *in vitro*. This effect was observed only in those five GH-secreting adenomas, that only partially responded to octreotide *in vivo*. The same inhibition was achieved with equimolar combination of BIM-23197 and BIM-23268 in these tumours. However, in five adenomas, well responding to octreotide *in vivo*, no additive effect of SSTR5 stimulation was found. Importantly, in the five partially octreotide responding adenomas the SSTR5 preferential analogue BIM-23268 was equally, or slightly more potent than BIM-23197, binding preferentially to SSTR2. Our results thus well correspond with these findings demonstrating existence of three types of GH-secreting pituitary adenomas. In the first type the GH-suppressive effect of somatostatin is mediated only through the SSTR2 subtype and the SSTR5 subtype stimulation has no additive effect. In the second type the GH-suppressive effect of somatostatin is mediated through both the SSTR5 and SSTR2 subtypes and maximal effect can be achieved by stimulation of both these receptors. The third type represent adenomas that do not respond to somatostatin inhibition at all. No differences were observed between these three groups in clinical and neuroradiological characteristics of the patients or histopathological classification of the adenomas. Somatostatin exerts its effect on PRL secretion through SSTR5 subtype in foetal pituitary cultures (Shimon et al. 1997a) and prolactinomas (Shimon et al. 1997b). In human prolactinomas, RT-PCR quantitative analysis showed a large predominance of SSTR5 *versus* SSTR2 mRNA and the SSTR1 transcript was also highly expressed (Jaquet et al. 1999). Our finding of higher potency of SSTR5 preferentially binding analogue B in comparison with the analogue A with the preference to SSTR2, as well as no additive effect of their combination, corresponds with these finding and is in keeping with previous results in prolactinomas (Shimon et al. 1997b; Jaquet et al. 1999) and mixed GH and PRL secreting acromegalic adenomas (Jaquet et al. 2000; Saveanu et al. 2001).

Our study demonstrates that adenomas, where the GH-suppressive effect of somatostatin is mediated through both the SSTR5 and SSTR2 subtypes and maximal effect can be achieved by stimulation of both these receptors are relatively common (three of ten unselected patients). New analogues with affinity to both SSTR2 and SSTR5 would be probably most potent in this patients' group.

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