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Expression of Somatostatin Receptors in Human Melanoma Cell Lines: Effect of Two Different Somatostatin Analogues, Octreotide and SOM230, on Cell Proliferation

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Somatostatin analogues (SAs) are potential anticancer agents. This study was designed to investigate the expression of somatostatin receptors (SSTRs) in melanoma cells and the effect of two SAs on cell proliferation and viability. Eighteen primary and metastatic human cutaneous melanoma cell lines were treated with octreotide and SOM230. Expression of SSTR1, SSTR2, SSTR3 and SSTR5 was assessed by real-time polymerase chain reaction. Proliferation, viability and cell death were assessed using standard assays. Inhibition was modelled by mixed-effect regression. Melanoma cells expressed one or more SSTR. Both SAs inhibited proliferation of most melanoma cell lines, but inhibition was < 50%. Neither SA affected cell viability or induced cell death. The results suggest that melanoma cell lines express SSTRs. The SAs investigated, under the conditions used in this study, did not, however, significantly inhibit melanoma growth or induce cell death. Novel SAs, combination therapy with SAs and their anti-angiogenic properties should be further investigated.

KEY WORDS: Malignant melanoma; Somatostatin analogues; Octreotide; SOM230; Cell culture; Somatostatin receptors; Cell proliferation; Apoptosis

Introduction

Besides inhibiting the release of growth hormone and affecting other eccrine activities, native somatostatin and synthetic somatostatin analogues (SAs) inhibit cell proliferation through cell cycle control and induction of apoptosis.[1,2] These actions are mediated via five specific somatostatin receptors (SSTR 1, SSTR 2, SSTR 3, SSTR 4 and SSTR 5).[1,2] Expression of SSTRs in neoplasms has raised the possibility of employing SAs as anticancer agents.[1 – 6] Nevertheless, with the

exception of neuroendocrine tumours,[4,7,8] their role as antineoplastic drugs remains unclear.[4,9]

Disseminated malignant melanoma presents a therapeutic challenge because it is highly resistant to current forms of therapy.[10,11] Data regarding the utility of SAs in melanoma are scarce.[12 – 15] The aim of the present study was to assess the *in vitro* effect of two SAs with different SSTR binding profiles, octreotide and SOM230,[1,2,16 – 19] on the proliferation and viability of melanoma cell lines, and to relate these results to the expression of their target receptors (SSTR 1, SSTR 2, SSTR 3 and SSTR 5) as measured by real-time polymerase chain reaction (PCR). As neither octreotide nor SOM230 bind to SSTR4, the expression of SSTR4 was not investigated.

Materials and methods

MALIGNANT MELANOMA CELL LINES AND CELL CULTURE

A total of 18 human skin malignant melanoma cell lines were obtained from four primary and 14 metastatic (12 cutaneous and subcutaneous and two visceral) tumours by one of the authors (RV).[20,21] The cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco® Cell Culture, Barcelona, Spain) with 10% fetal calf serum (FCS; Gibco®) and 20 mM penicillin/streptomycin/glutamine (Sigma–Aldrich, Madrid, Spain) at 37 °C and in 5% CO_2.

The cells were trypsinized and plated in 96-well plates and incubated with octreotide or SOM230 (Novartis, Basel, Switzerland) for 1, 3 and 6 days. As 25 µg/ml octreotide was previously shown to be the best inhibitory concentration for neoplastic cells,[22] octreotide was added to the cell cultures at concentrations of 10, 25 or 50 µg/ml (9.81, 24.53 or 49.05 µmol, respectively). The SOM230 was added to cell cultures at concentrations of 0.01, 0.10 or 1.00 µmol, as suggested by Novartis. Control cells for each experiment were incubated in DMEM with 10% FCS. All treatments were performed in triplicate and repeated at least three times.

The effect of each SA on melanoma cell proliferation was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay (Sigma–Aldrich) as previously described.[20] The effect of each SA on cell death and viability was determined by the measurement of lactate dehydrogenase (LDH) activity in the cells using a CytoTox 96® kit (Promega, Madison, WI, USA), according to the manufacturer's instruction. Cell death ratios were obtained by referring the LDH values in the medium of a SA-treated culture to the total LDH content of its corresponding control culture. The mean percentage cell death found in untreated cultures was calculated and subtracted from the final death ratios. Apoptosis was evaluated by nuclei staining with Hoescht 33258 (bis-benzimide; Sigma–Aldrich) in the M14 cell line, which was the most responsive cell line to both SAs.

REAL-TIME PCR ANALYSIS OF SSTR EXPRESSION

Total cellular RNA was extracted using TRIzol® reagent (Life Technologies™, Carlsbad, CA, USA) and 1 µg of total RNA was reverse transcribed (RT) into cDNA using TaqMan® Reverse Transcription Reagents (part No. N808-0234; Applied Biosystems, Foster City, CA, USA). The RT reaction was then used as a template for a 25 µl reaction for real-time PCR detection of human somatostatin receptors using TaqMan® Technology on a ABI Prism® 7000 sequence detection system (both from Applied Biosystems). Applied Biosystems' Assays-on-Demand™ primers and TaqMan® MGB probes (FAM™ dye-labelled reporter and no

fluorescent quencher) for all four target genes and pre-developed glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (VIC® dye-labelled probe) were used.

Quantification of gene expression was performed in separate tubes (Singleplex) for both the target genes and the endogenous control gene using the commercially-available primer and probe sequences for human somatostatin receptors SSTR1, SSTR2, SSTR3 and SSTR5 and GAPDH (Applied Biosystems; assay identification numbers HS 00174949m1, HS 00265617s1, HS 00265624s1, HS 00265639s1 and HS 99999905m1, respectively). The thermal cycling conditions were uracil-*N*-glycosylase activation for 2 min at 50 °C and AmpliTaq® DNA polymerase activation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min.

Duplicate control values from two independent RNA extractions were analysed with Quantitative Relative Software (Applied Biosystems) using the comparative CT ($\Delta\Delta Ct$) method as described by the manufacturer.[23] The amount of target ($2^{-\Delta\Delta Ct}$) was obtained by normalizing to an endogenous reference gene (*GAPDH*) and relative to a calibrator melanoma cell line, M20, to give the relative quantity (RQ) value. The M20 melanoma cell line was selected as the calibrator because it had SSTR expression levels closest to the median SSTR expression levels.

STATISTICAL ANALYSIS

The statistical analysis included computation of the Pearson correlation coefficient, the Wilcoxon's rank sum test, and mixed-effect model estimations. The latter were fitted to the data to take into account the variability explained by repeated experiments in each cell line (random effect). Thus, initially, the observed inhibition induced by each treatment was modelled separately, depending on time (in days, included as a polynomal function), concentration (included as a factor), and their interaction. Then, SSTR specific gene expressions were included once dichotomized by median value. A constant effect was assumed for the SSTR values, independent of time and concentration. Concentration and SSTR dummy effects were estimated as referred to the lowest category, and the likelihood ratio test was used to measure the SSTR expression contribution to the multivariate model. A P-value < 0.05 was considered to be statistically significant. Analysis was performed with 'R', the language and environment for statistical computing developed by the R Development Core Team (created by Ross Ihaka and Robert Gentleman, University of Auckland, New Zealand).

Results

Treatment of melanoma cell lines with each SA for 1, 3 or 6 days inhibited cell proliferation in a concentration-dependent manner (Fig. 1). The highest inhibitory response was observed at day 3 and with the highest concentration of both treatments, with a mean inhibition of control proliferation of 21.54% with octreotide and 26.92% with SOM230 (Table 1). The highest mean inhibition of proliferation in a melanoma cell line was 39.2% (observed in M14 and M20) with octreotide 49.05 µmol treatment for 3 days and 49.2% (observed in M38) with SOM230 1.00 µmol for 3 days (Fig. 2). Mean inhibitory responses to octreotide and SOM230 were not statistically different according to Wilcoxon's rank sum test with continuity correction. As detected by the LDH cytotoxicity assay, neither SOM230 nor octreotide modified cell

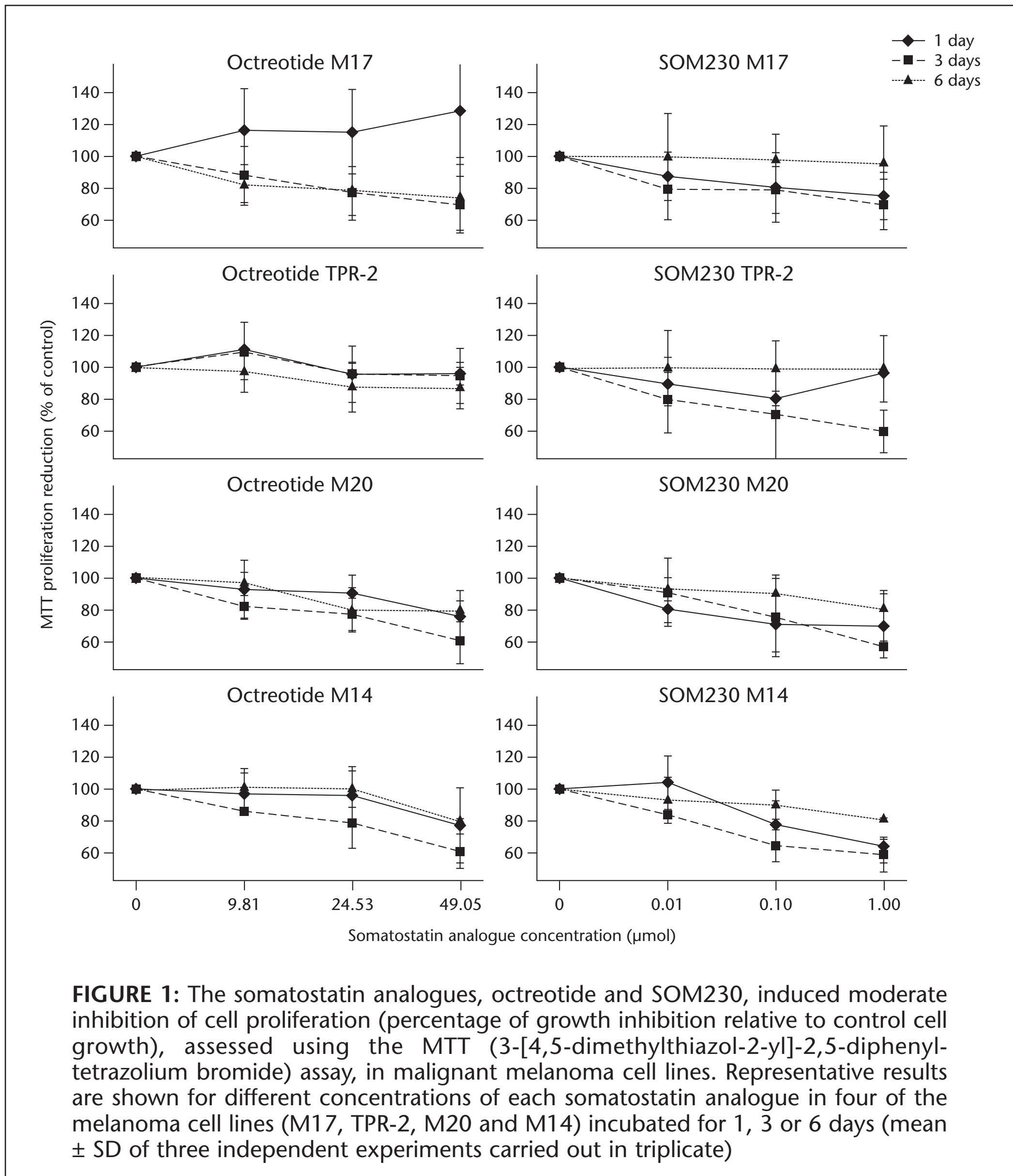

FIGURE 1: The somatostatin analogues, octreotide and SOM230, induced moderate inhibition of cell proliferation (percentage of growth inhibition relative to control cell growth), assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay, in malignant melanoma cell lines. Representative results are shown for different concentrations of each somatostatin analogue in four of the melanoma cell lines (M17, TPR-2, M20 and M14) incubated for 1, 3 or 6 days (mean ± SD of three independent experiments carried out in triplicate)

viability or were toxic (data not shown). Additionally, incubation of one of the most SA responsive melanoma cell lines (M14) with the highest concentration of each SA for 3 days did not induce an apoptotic morphology (Hoechst 33258 staining, data not shown).

Analysis of the expression of SSTRs by real-time PCR demonstrated that all melanoma cell lines tested expressed at least one of the four SSTRs studied (Fig. 3). The SSTR expressed at the highest levels in the majority of cell lines tested was SSTR 2, followed by SSTR 1, SSTR 3 and SSTR 5. Some cell lines showed higher expression of other SSTRs; in particular, five cell lines expressed

TABLE 1:
Observed and estimated mean percentage of inhibition of proliferation induced by treatment of malignant melanoma cell lines with the somatostatin analogues, octreotide and SOM230

	Octreotide								SOM230	
	Unadjusted by SSTR		Adjusted by SSTR1		Adjusted by SSTR2		Adjusted by SSTR3		Unadjusted by SSTR	
Duration of treatment, drug dose	Observed (%)	Estimated (%)	Low[a] (%)	High[a] (%)	Low[a] (%)	High[a] (%)	Low[a] (%)	High[a] (%)	Observed (%)	Estimated (%)
Day 1, CI	0.53	0.14	4.72	–4.44	2.15	–1.87	–1.46	1.74	3.96	5.07
Day 1, CII	2.53	1.91	1.91	–7.24	1.91	–2.12	1.91	5.11	10.21	3.95
Day 1, CIII	5.49	6.22	6.22	–2.93	6.22	2.20	6.22	9.43	15.18	10.18
Day 3, CI	10.92	13.77	15.14	12.39	12.65	14.88	9.58	17.95	10.62	13.44
Day 3, CII	16.97	15.53	12.33	9.58	12.40	14.64	12.95	21.32	16.93	12.32
Day 3, CIII	21.54	19.85	16.65	13.90	16.72	18.96	17.27	25.63	26.92	18.55
Day 6, CI	–0.20	–2.66	–6.08	0.77	–8.47	3.16	0.74	–6.05	–1.04	–4.97
Day 6, CII	–2.52	–0.89	–8.89	–2.04	–8.71	2.91	4.11	–2.68	–1.74	–6.09
Day 6, CIII	2.89	3.43	–4.58	2.28	–4.40	7.23	8.43	1.63	2.00	0.14

[a]Estimated values for the percentage of inhibition induced by octreotide depending on the corresponding somatostatin receptor (SSTR) expression level (low versus high).
CI, concentration 1 (octreotide 9.81 µmol or SOM230 0.01 µmol); CII, concentration 2 (octreotide 24.53 µmol or SOM230 0.10 µmol); CIII, concentration 3 (octreotide 49.05 µmol or SOM230 1.00 µmol); SSTR, somatostatin receptor.

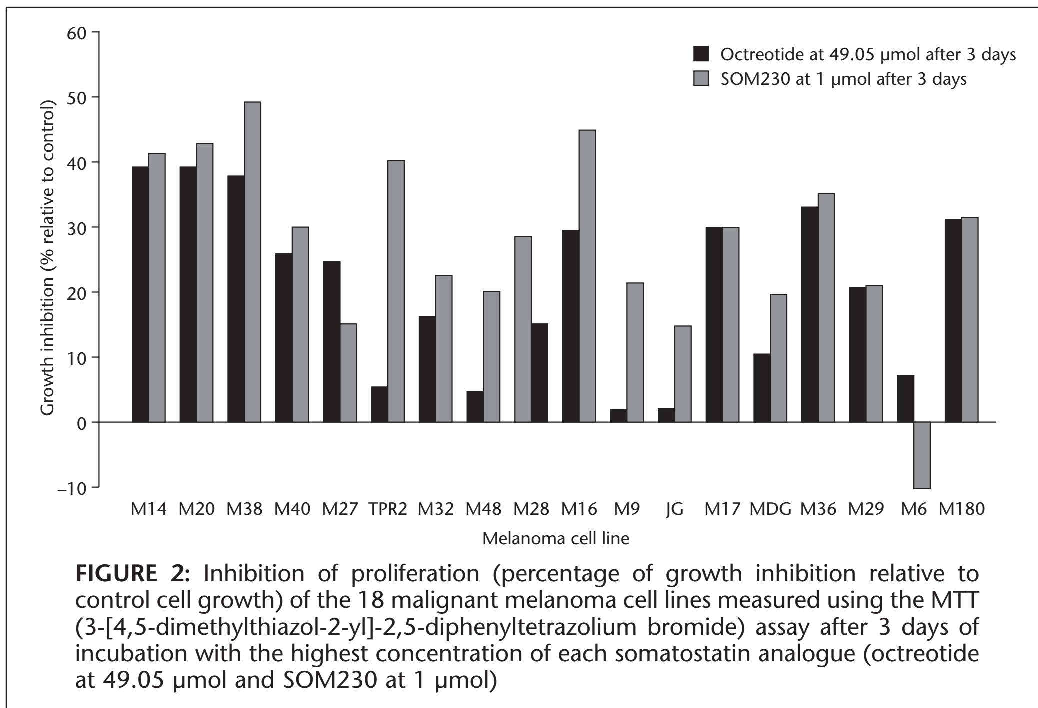

FIGURE 2: Inhibition of proliferation (percentage of growth inhibition relative to control cell growth) of the 18 malignant melanoma cell lines measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay after 3 days of incubation with the highest concentration of each somatostatin analogue (octreotide at 49.05 μmol and SOM230 at 1 μmol)

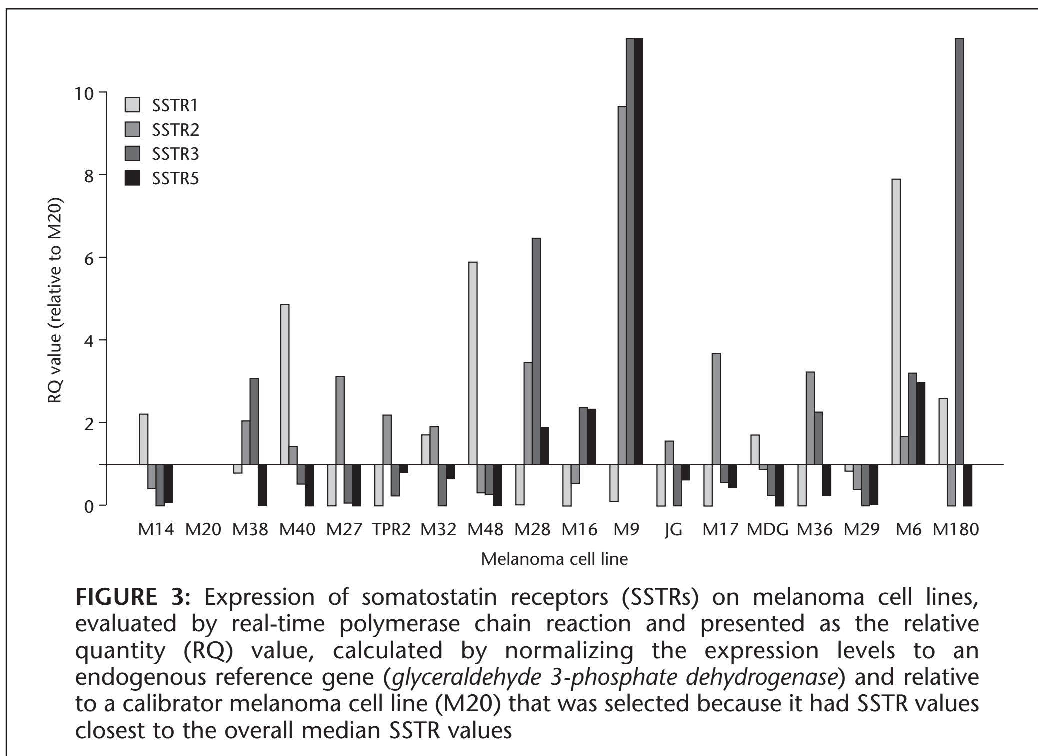

FIGURE 3: Expression of somatostatin receptors (SSTRs) on melanoma cell lines, evaluated by real-time polymerase chain reaction and presented as the relative quantity (RQ) value, calculated by normalizing the expression levels to an endogenous reference gene (*glyceraldehyde 3-phosphate dehydrogenase*) and relative to a calibrator melanoma cell line (M20) that was selected because it had SSTR values closest to the overall median SSTR values

SSTR 3 with RQ values ≥ 3 relative to the M20 calibrator median level (with RQ up to 68.64 for M9 and 90.13 for M180). Only M6 expressed all four SSTRs above the median expression level. The M9 cell line showed the highest levels of expression for all SSTRs except for SSTR1. Levels of expression of all SSTRs were positively correlated with each other, except for SSTR 1 which showed small but statistically significant negative correlations with SSTR 2 (r = –0.35) and SSTR 5 (r = –0.15), and no significant linear correlation with SSTR 3. The highest positive correlation coefficients were shown for SSTR 2 versus SSTR 5 (r = 0.85) and SSTR 3 versus SSTR 5 (r = 0.56).

The modelling included the melanoma cell line as a random effect and time, concentration and expression of SSTRs as fixed effects. Table 1 shows the estimated values derived from five different models. The first and last columns in Table 1 show the models where the overall estimation of inhibition for each treatment did not take into account the expression of SSTRs (i.e. unadjusted by SSTR). The other three columns give the results from the models that show a significant SSTR expression contribution in explaining the response to octreotide (no significant contribution was found for SOM230). Thus, there was a statistically significant contribution from SSTR1, SSTR2 and SSTR3 on the response to octreotide, depending on time. The melanoma cell line random effect had a high impact on the response to SOM230 treatment, where variability within melanoma cell lines represented 18% of the total variability compared with 9% obtained with octreotide.

According to the unadjusted models, the maximum mean inhibition was predicted for day 3 with the maximum concentration of both treatments, with a predicted mean inhibition of 19.85% and 18.55% for octreotide and SOM230, respectively (Table 1). Taking into account the influence of the expression of SSTRs in the cell lines' responses to octreotide, the results showed that, with the highest octreotide concentration and after 3 days of treatment, high expression levels of SSTR1 were associated with a lower predicted mean inhibition (13.90%) than the overall fitted mean of 19.85%. In contrast, with the same time and concentration conditions, high expression of SSTR3 was associated with the highest predicted mean inhibition (25.63%). In reference to the expression of SSTR2, the biggest differences were seen not on day 3 (when fitted inhibition with the highest concentration of octreotide was similar to the overall response) but on day 6, where the melanoma cell lines with low expression of SSTR2 showed no inhibition compared with the melanoma cell lines with high expression of SSTR2. Inhibition data in cell lines were well fitted by the different models except for the inhibition effect of octreotide in M16 and the inhibition effect of SOM230 in M48.

Discussion

The results from the present study are in agreement with previous studies showing that SSTR2 is the most abundantly expressed SSTR in the majority of tumour types[17] and in cutaneous and uveal melanoma.[12,13,17,24] Melanoma expression of SSTRs has been shown to be variable in different studies, probably due to the diversity of materials and methods employed (tissue, cell lines, RT–PCR, Western blot, immunohistochemistry).[12,13,17,24] The present study was the first to evaluate SSTR expression quantitatively by real-time PCR in a large number of melanoma cell lines.

Both of the SAs used in the present study

inhibited melanoma cell proliferation in a concentration-dependent manner. This inhibition did not, however, exceed 50% of the control proliferation under any conditions (Table 1, Fig. 2). SOM230 inhibited control proliferation more than octreotide, although there were no statistically significant differences between the two SAs. It is worth mentioning, however, that the employed concentration of octreotide was 50 – 1000-fold higher than that of SOM230. To date, the authors have not been able to identify any published experiments that could be compared with the present study, as previous studies have used different SAs (vapreotide, lanreotide, TT232 and RC121), different octreotide concentrations or different assay conditions.[12 – 15,24,25] Although it has been argued that SOM230, acting through SSTR1 and SSTR3, could be useful in SSTR-positive malignancies,[1] no published reports on SOM230 and melanoma have been identified. The highest mean inhibition of proliferation in the present study occurred on day 3 in response to the highest concentration of each SA, but inhibition was generally decreased on day 6 compared with day 3, with time apparently having a quadratic rather than linear effect (Fig. 1). The inhibitory effect of each SA appeared to reach a peak at 1 – 3 days, as described previously for colon cancer cells.[22]

Attempts to analyse the correlation between SSTR expression and the response of melanoma cells to the SAs used were difficult. In total, 18 malignant melanoma cell lines, four SSTRs with potentially different effects on cell cycle and apoptosis, and two SAs with different binding profiles were used in the present study, all of which could have led to a range of abnormal responses to SSTR stimulation. Both of the SAs used in the present study are known to bind with high affinity to SSTR2 and SSTR5.[1,2,16 – 19] Octreotide binds moderately to SSTR3 and hardly at all to SSTR1, whereas SOM230 shows high affinity to SSTR3 and very high affinity to SSTR1.[1,2,1 – 19] Given so many sources of variability, it seems reasonable that the present study did not show any clear statistical relationship between the expression levels of the SSTRs studied and the response to SOM230 or to octreotide.

Although the inhibitory effect of each SA on melanoma cell proliferation was only modest (< 50% of the control proliferation), a statistical model for the inhibition response to SA by concentration, time and expression of SSTRs was designed that fitted the behaviour of almost all cell lines with the exception of one cell line for each SA. A long list of possible explanations for the abnormal behaviour of SSTR-positive neoplastic cells in response to SA treatment has been presented, reflecting abnormalities in the pathways involved in the response to SA, from down-regulation of SSTR affinity to mutations in SSTR genes.[18] To the authors' knowledge, this type of study has not been done in malignant melanoma.

In summary, the present study was the first to assess quantitatively the expression of SSTRs (1, 2, 3 and 5) by real-time PCR in a range of malignant melanoma cell lines. SSTR2 was the SSTR with the highest overall level of expression. Both SAs, octreotide and SOM230, inhibited the proliferation of melanoma cells in a concentration-dependent manner. This response was consistently predicted by a statistical model. The inhibition induced by both of the SAs did not, however, exceed 50% of control cell proliferation under any conditions. Moreover, neither of the SAs was able to modify cellular viability or induce cell death. Thus, octreotide and SOM230 are probably

not potential antineoplastic drugs for metastatic melanoma, at least when used as single agents. As SSTR expression has been clearly demonstrated in malignant melanoma, here and by other authors, and as at least some of the SSTRs expressed by melanoma cells, such as SSTR2 and SSTR3, can induce apoptosis,[26,27] the use of SAs as anticancer agents in melanoma remains a possibility. Novel SAs that are better able to induce apoptosis,[15,28] the use of SAs in combination with chemotherapy, radioactive or other cytotoxic agents,[2,4,25,27] and their anti-angiogenic properties[24,29,30] should be further investigated.

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Conflicts of interest

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