Differential efficacy of SSTR1, -2, and -5 agonists in the inhibition of C6 glioma growth in nude mice

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SOMATOSTATIN (SST), through the activation of a family of five G protein-coupled receptors [SST receptor (SSTR)1–5] (46), exerts antiproliferative effects in normal and tumor epithelial and endocrine cells (25, 51), thus providing the basis for the clinical use of SST analogs for the treatment of pituitary adenomas (13) and neuroendocrine tumors (28). SSTRs are expressed in several human cancer cells, including neuroendocrine, gastroenteropancreatic, brain, prostate, lung, and breast tumors; more than one subtype was detected in each tumor histotype, with SSTR2 being the most frequently expressed subtype (54).

Although both direct (induction of cell cycle arrest or apoptosis) (14) and indirect [antiangiocrine (60) and antiangiogenic (2)] antitumor activities of SST were identified, its possible clinical application as anticancer drug is still controversial (63).

Multiple intracellular mechanisms Have been involved in the antiproliferative effects of SST (20). SST indirectly regulates tumor proliferation, inhibiting the release of growth-promoting hormones and growth factors (growth hormone, insulin-like growth factor I, basic fibroblast growth factor) (38, 40) or proangiogenic factors (vascular endothelial growth factor) (43). SST directly inhibits angiogenesis acting on both monocytes through the production of a number of cytokines and growth factors for endothelial cells (2) and on endothelial cell proliferation via the inhibition of both extracellular signal-regulated kinase 1/2 (ERK1/2) and endothelial nitric oxide synthase activities (5, 23).

The intracellular signaling pathways mediating the direct regulation of tumor cell proliferation by SST have been studied extensively (3, 18, 22, 26, 27, 57).

SST antiproliferative activity was related to the inhibition of adenylyl cyclase, the modulation of ion currents, the activation of phosphotyrosine phosphatases (PTP), and the regulation of mitogen-activated protein (MAP) kinases (20). In particular, the modulation of PTP activity by SST is now regarded as a major mechanism in the regulation of cell growth (12, 24, 27, 50, 53). PTPs, a receptor-like PTP involved in the inhibition of cell growth and inducing cell differentiation (37, 48), was reported to be required for the SST-dependent cytostatic activity in thyroid (22) and glialoma cell lines (42) as well as in primary cultures of human glioblastoma (GBM) cells (41). In addition, SST increases PTP activity in C6 cells, causing a direct interaction of this PTP with ERK1/2 and the dephosphorylation and inactivation of this MAP kinase in vitro (41). Interestingly, in C6 and Chinese hamster ovary-K1 cells, SSTR1-mediated activation of PTPs involves the formation of a multieffector complex bound to the receptor, causing the G protein-dependent sequential activation of tyrosine kinases (JAK2, Src) and PTPs (short heterodimer partner-2) (6). Similar, although distinct, mechanisms were identified in other cell types via SSTR2 (18), so that raised the possibility that this interplay between tyrosine kinases and PTP may represent a modular mechanism of signal transduction mediated by SSTRs (21). Finally, the inhibition of ERK1/2 induced by SST via PTP activity results in the overexpression of p27Kip1, a cyclin-
dependent kinase (CDK) inhibitor that negatively regulates G1/S transition (22, 35, 42, 49).

However, all these in vitro results do not necessarily imply that similar antitumoral effects of SST can be reproduced in vivo. In fact, cells in culture are not subjected to the same environment occurring within the tumor mass, and the cellular and molecular mechanisms in vitro may not always reflect the same major function in vivo. Furthermore, differences between in vitro and in vivo antiproliferative effects of drugs have sometimes been observed.

In this study, we evaluated the effectiveness of SST and selective SSTR agonists on the proliferation of glioma cells xenotransplanted in nude mice. Indeed, most data about the effect of SST analogs on glioma cells have been obtained from in vitro experiments, and to date no studies have approached the intracellular mechanisms activated by SSTRs in vivo.

Among brain tumors, high-grade (WHO IV) gliomas (GBM) are poor-prognosis, histologically heterogeneous tumors characterized by tumoral cells, stroma, inflammatory infiltrates, and vascular structures. Rat glioma cell models, when used in vivo experimental settings, resemble with a sufficient approximation human high-grade brain tumors (for example, as far as neoangiogenic properties) and thus may provide useful information on in vivo responses to novel therapeutic approaches. The highly tumorigenic rat C6 glioma cell line has been widely used in both in vivo and in vitro studies for many years as a experimental model for the study of GBM growth, invasion, and neovascularization (31). Importantly, recent data demonstrated the presence of tumor-initiating cells, also known as cancer stem cells (CSCs), in cultured C6 cells (58). CSCs, a small tumor cell subpopulation endowed of self-renewal properties, are believed to be responsible for initiation and progression of tumors. In this view, only CSCs are able to reproduce the neoplasia when tumor cells are xenografted into immunodeficient mice. CSCs have been identified, characterized, and isolated from human GBM and established brain tumor cell lines (30, 59). Similarly to the human counterpart, C6-derived CSCs display characteristics of self-renewal, potential of multilineage differentiation in vitro, and tumorigenic capacity in vivo (39, 65). These observations further support the relevance of this cell line as a model for the study of human tumors.

On this basis, in the current study we tested SST antiproliferative activity in rat C6 glioma cells xenografted in nude mice and the intracellular pathways involved. We previously reported that C6 cells respond in vitro to SST treatment with an increase in PTP activity and a decrease of the proliferation rate (41). Thus, since it is not possible to analyze PTP activity in vitro, we performed immunohistochemical analysis of C6 experimental gliomas treated with vehicle, SST, or SST analogs to evaluate the regulation of downstream effectors of this PTP as identified in vitro (9, 22, 41), namely the inhibition of ERK1/2 and the upregulation of p27kip1.

Moreover, since tumor cells express multiple SSTRs, it is essential to deepen the individual role of the different SSTR subtypes in the regulation of cell proliferation and intracellular pathways. We recently reported that C6 cells natively express SSTR1, -2, -3, and 5, but SSTR3 was devoid of antiproliferative activity (9). Thus we analyzed the role of individual SSTRs and their possible interactions, evaluating the efficacy of SSTR-selective agonists as antitumoral agents against C6 gliomas in vivo. In particular, we investigated the efficacy of BIM-23745 (SSTR1 selective), BIM-23120 (SSTR2 selective), and BIM-23206 (SSTR5 selective). Moreover, the modulation of the activity of each receptor subtype induced by the co-stimulation of SSTRs was analyzed by using the bispecific molecules BIM-23704 (SSTR1 and -2) and BIM-23190 (SSTR2 and -5).

MATERIALS AND METHODS

Cell culture and reagents. C6 rat glioma cell line was obtained from the Interlab Cell Line Collection (Genoa, Italy). Cells were maintained in Ham’s F-12 medium supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin, and streptomycin (100 U/ml) (all from Euroclone, Milan, Italy).

SST14 was from Calbiochem (Lucerne, Switzerland), SST agonists BIM-23745, BIM-23120, and BIM-23206, and bispecific analogs BIM-23190 and BIM-23704 were provided by Biomeasure/IPSEN (Milford, MA).

Individual binding characteristics of these compounds were reported previously (9). Briefly, SST has high affinity for all receptor subtypes; BIM-23745 shows high binding affinity only for SSTR1 (IC50: 42 nM), BIM-23120 is selective for SSTR2 (IC50: 0.3 nM), and BIM-23206 binds only SSTR5 with high affinity (IC50: 2.4 nM). The affinity of these SST analogs for SSTR3 and -4 are negligible. BIM-23190 is a bispecific analog with high affinity and selectivity for both SSTR2 and SSTR5 (IC50: 0.34 and 11.2 nM, respectively), BIM-23704 is selective for SSTR1 (IC50: 6.3 nM) and SSTR2 (IC50: 1.4 nM).

All other reagents were from Sigma-Aldrich (Milan, Italy), unless reported otherwise.

Animals. Male athymic nude (nu/nu) mice, 5–6 wk old (Charles River, Milan, Italy), were housed in pathogen-free standardized environmental conditions at 25°C with a 14:10-h light-dark cycle with free access to food and water. Animals were handled in accordance with the institutional animal care guidelines. All experiments were approved by the Ethics Committee for Animal Use in Cancer Research at the National Institute for Cancer Research (Genoa, Italy).

Experimental protocols. Exponentially growing C6 cells (3 × 10⁵) were harvested, checked for viability (>90%), mixed with liquid Matrigel (DB Biosciences, Milan, Italy) to a final volume of 250 μl at 4°C, and injected subcutaneously (sc) into the flanks of nude mice (day 0) as reported (23). After 24 h (day 1), mice were randomized into different groups of nine animals each and treated twice daily with doses of 50 μg of each tested drug, following the different experimental protocols.

In the experimental protocol 1, the first group received sc pertumor saline solution, and the second one received SST until day 19. In experiment 2, mice were assigned to five experimental groups: group 1, control mice received saline solution; group 2, mice were given the analog BIM-23745; group 3, mice were injected with BIM-23120; group 4, mice received BIM-23206; and group 5, mice received SST until day 19. In experiment 3, mice were divided into seven experimental groups: group 1, mice were given the saline solution; group 2, mice were injected with BIM-23120; group 3, mice received BIM-23206; group 4, mice were injected with BIM-23745; group 5, mice received the bispecific analog BIM-23A704 (SSTR1 and -2); group 6, mice were treated with BIM-23A190 (SSTR2 and -5 selective agonist); and group 7, mice received SST as reference group. During all experiments, tumor volume was monitored every 2 days by measuring tumor diameters using calipers, and the tumor volume was calculated using the following formula: V (mm³) = (D × d²)/2, where d (mm) and D (mm) are the smallest and largest perpendicular tumor diameter, respectively (23). Systemic toxicity was evaluated recording changes in body weights and behavioral symptoms every 2 days. After 20 days, the animals were euthanized and the tumors removed, weighed, and fixed in formalin for histological examination. All of the experiments were repeated twice. In a different experiment series,
tumors treated with SST or vehicle were collected at different time points (5, 8, 12, and 15 days) to evaluate the progressive effects of the treatments on ERK1/2 phosphorylation.

**Immunohistochemistry.** Immunohistochemical detection of the relevant proteins was performed on tumor sections (4 μm) of paraffin-embedded tissue stained with the appropriate antibodies, as reported (9). Briefly, deparaffinized and rehydrated sections were heated in 0.1 M citrate buffer (pH 6.0) for antigen retrieval. After cooling (room temperature for 10 min) the sections were permeabilized in PBS 0.3%-Triton X-100 for 10 min, washed in TBS (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.6), saturated for 30 min in 10% normal goat serum (in TBS), and incubated overnight at 4°C with appropriate dilution of primary antibodies in CHEM-MAT solution (Dako, Glostrup, Denmark). The following antibodies were used: rabbit anti-phospho-ERK1/2 (dilution 1:100; Cell Signaling Technology, Beverly, MA), mouse anti-p27<sup>kipl</sup> (dilution 1:100; Transduction Laboratories, Lexington, KY), and mouse anti-Ki-67 (dilution 1:25; Dako). After washes in TBS, sections were incubated with biotinylated secondary antibodies anti-mouse (dilution 1:400; Dako) or anti-rabbit (dilution 1:500; Dako) for 30 min in TBS. Finally, the sections were washed and incubated for 20 min with Strept-AB Complex-AP according to the manufacturer’s instructions (Dako), revealed with BCIP-NBT-INT substrate system (Dako), and counterstained with hematoxylin before being mounted with Mowiol (Calbiochem).

Ki-67 (MIB-1), phospho-ERK1/2, and p27<sup>kipl</sup>-positive cells were evaluated by visual inspection, and a labeling index (LI) was calculated as a percentage of positive cells/total number of tumor cells counted (≥500) in five randomly selected microscopical fields from each tumor section.

Immunofluorescence (IF) staining was performed using the cleaved caspase-3 (Asp175) monoclonal antibody (Cell Signaling Technology) diluted 1:100. Sections were labeled with 1:100 fluorochrome-conjugated secondary antibody Alexa fluor 568 (Molecular Probes; Invitrogen, Milan, Italy) in the dark for 1 h. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Slides were then stained with Sudan Black B (0.3% in 70% EtOH) to avoid tissue autofluorescence (8), washed with TBS, and mounted with Mowiol. IHC and IF negative controls were included in all the immunohistochemical analyses by omitting the primary antibodies.

Immunohistochemistry (IHC) and IF slides were visualized and photographed with a DM2500 microscope (Leica Microsystems, Wetzlar, Germany) equipped with a DFC350FX digital camera (Leica Microsystems) and blindly scored by two researchers in independent evaluations. The overall interobserver variation was <7% (7).

**Evaluation of intratumoral microvascular density.** Tumor sections were stained by using the biotin-streptavidin peroxidase system followed by hematoxylin counterstain, as described below. Immunohistochemical staining was performed with mouse monoclonal CD31 and CD34 antibodies (Ventana Medical Systems, Illkirch, France) with the highest sensitivity and specificity for staining microvessels (61). CD31 targeting the platelet endothelial cell adhesion molecule 1 is detected in endothelial cells ranging from non-lumen-forming cells to lumen-forming cells. Anti-CD34 antibody targets the transmembrane sialo-protein in precursors of undifferentiated endothelial cells to differentiated endothelial cells. Negative controls were included in all experiments. For the evaluation of the intratumoral microvessel density, all independent CD31/CD34-positive structures were counted as an individual vessel irrespective of the presence of an identifiable lumen according to the Weidner method (64) and an international consensus report (61).

A DM2500 microscope (Leica Microsystems) equipped with a charge-coupled device camera was used. Since microvessels are distributed heterogeneously throughout the tumor, the screening of the slides was done by the hotspot method: the whole sections were initially scanned at low magnification (×10) to identify the areas with highest number of microvessels (hot spots). The count of microvessels in five sufficiently extended fields (×40) was then performed. The mean microvessel count of the five most vascular areas was taken as the microvessel density, which was expressed as the absolute number of microvessels for a sample. The number of CD31- and CD34-positive structures in each field was independently counted by two observers, and the overall interobserver variation was <10%.

**Statistical analysis.** Experiments were independently repeated twice, and the statistical significance of differences in tumor masses and between groups was evaluated using one-way ANOVA test followed by the Neumann-Keuls comparison. All values are expressed as means ± SE. A P value equal to or <0.05 was considered statistically significant.

**RESULTS**

**Effects of SST on glioma development in mice in vivo.** To analyze the antiproliferative effects of SST in vivo, C6 cells were injected sc in the flank of nude mice in a basement membrane attachment matrix (Matrigel) to enhance engraftment rate, which was >95%. Treatment with SST (50 μg sc peritumoral, twice/day) or vehicle (control group) was started 24 h after C6 cell injection and continued for 19 consecutive days. Tumor volume was recorded every 3 days, and growth curves are shown in Fig. 1A.

Administration of SST significantly reduced C6 xenograft tumor growth, since tumor volumes shrank markedly. This effect was statistically significant starting from day 15 (P < 0.05) of treatment, and the differences between tumors treated with saline or SST progressively increased up to the end of the experiment, reaching a highly significant (P < 0.01) reduction on day 17 compared with controls. At the end of the observational period (day 19), the mean volume of the SST-treated tumors was about threefold smaller than the vehicle-treated ones (P < 0.001).

The significant slowing down of tumor growth induced by SST was confirmed, evaluating the weights of tumors explanted after the euthanization of the mice. On day 20, SST-treated tumors showed a significantly lower (>60%) tumor weight compared with saline-treated tumors (Fig. 1B).

To evaluate the potential toxicity of SST treatment, tumor-bearing mice were observed over the course of the study and weighed every 3 days. All of the animals remained healthy, and no behavioral changes and no external toxic symptoms were noted. SST toxicity, as assessed by animal weight loss, was minimal in the SST-treated group (reduction <5%) compared with the saline-treated group (Fig. 1C).

**Effect of SST treatment on neovessel formation in C6 tumors.** Neangiogenesis is an absolute requirement for the growth of solid tumors, and C6 gliomas are characteristically highly vascularized tumors when they are inoculated in mice. SST effect on angiogenesis in C6 xenografts was observed in explanted tumors from the previous set of experiments by histological analysis after hematoxylin and eosin (H & E) staining to detect the presence and the distribution of the neovascularization process. Both SST-treated and untreated tumors showed a dense cellular structure, but the untreated ones displayed a net organization of small and large vessels, as demonstrated by H & E staining (Fig. 2A, left). In contrast, SST-treated neoplasms formed very undeveloped microvessels, showing a significant reduction in their density (Fig. 2A, right).

To obtain a more precise evaluation of this observation, sections from control and SST-treated tumors were stained...
with anti-CD34 and anti-CD31 antibodies to identify endothelial cells and quantify intratumoral microvessel densities (Fig. 3A). SST treatment significantly ($P < 0.01$) reduced the number of neovessel formation in C6 gliomas, inducing the presence of only very small endothelial structures (Fig. 3B).

**Effect of SST treatment on caspase-3 activation in C6 tumors.** To verify the possible activation of apoptotic process by SST in C6 tumors, we stained tumor sections with an antibody that specifically recognizes fragments (17/19 kDa) of cleaved (active) caspase-3 but not the full-length, inactive caspase-3. Cleaved caspase-3, a critical effector of apoptosis activated in response to most apoptosis triggers, is a biochemical marker easily detectable on paraffin-embedded sections (15). We determined cleaved caspase-3 immunoreactivity in conjunction with nuclear condensation detected by DAPI staining as a morphological evidence of apoptosis. The specific active caspase-3 antibody mainly stained the cytoplasm of cells, whose nuclear morphology was consistent with apoptosis, along with the cytoplasm of some morphologically healthy-looking cells, thus suggesting the presence of active protein at the early stage of apoptosis. Cleaved caspase-3 occasionally was also detected in nuclei, consistent with the reported translocation of the active protein into the nucleus. Untreated tumors showed large immunonegative areas, with some scattered single positive cells (<1% of the cells) distributed throughout the tissue. C6 xenografts treated with SST showed a small increase (5%) in immunopositive cells (Fig. 2C). However, in treated tumors, apoptotic cells were not uniformly distributed over the whole tissue sections but mostly clustered in areas around fragmented nuclei or as scattered cells within the section.

**Identification of SST intracellular effectors by IHC of C6 glioma tumors.** The direct antiproliferative effects by SST were analyzed by Ki-67 nuclear immunostaining of C6 cells in tumor sections to evidence actively proliferating cells. SST-treated tumors (Fig. 2A, right) showed a marked reduction of the level of proliferative activity, whereas saline-treated tumors showed a marked Ki-67 immunoreactivity (Fig. 2A, left). Thus SST was able to inhibit C6 cell proliferation in vivo, reducing proliferating cell fraction. Immunohistochemical results were quantified by counting positive cells in at least five random fields of sections from each removed tumor and plotted as percentage of immunopositive cells (LI). Figure 2B reports the histograms from LI scores for Ki-67, confirming the significant reduction in proliferating cells.

In vitro, SST controls C6 cell proliferation via the inhibition of ERK1/2 and the upregulation of p27kip1 (9); thus we evaluated the role of these pathways in vivo.

To this aim, we analyzed immunohistochemically the level of ERK1/2 phosphorylation/activation in the C6 tumor sections. In saline-treated tumors, the high proliferative activity was correlated with a powerful activation of ERK1/2, as shown by the density of cells stained with phospho-ERK1/2 antibody (Fig. 2A, left). Moreover, most of the phosphorylated ERK1/2 was observed in the nuclei of tumor cells, as expected for the proliferative effects of these kinases. Interestingly, although extremely diffuse throughout the tumor, active ERK1/2 was identified in discrete tumor areas, mainly at the periphery of the tumors, whereas in the center fewer immunopositive cells were detected, although an overall high level of activation was observed in all areas of the tumors (data not shown). In SST-treated tumors, an impressive reduction in ERK1/2 phosphorylation was observed. Indeed, although few positive cells were occasionally identified, it was not possible to identify in these sections large areas of cells containing active ERK1/2, as observed in the control tumors (Fig. 2A, right). Interestingly, the kinetics of SST effects on ERK1/2 phosphorylation was evaluated, in a different experimental setting, by repeated sampling of ERK1/2 at different time points (days 5, 8, 12, 15, and 19 of the treatment). No time-dependent significant modulation of ERK1/2 activation was observed (as far as % immunopositive cells/total cell number), but significantly lower immunopositive cells were present throughout the observation period in SST- than in vehicle-treated cells (data not shown).
of the CDK inhibitor p27Kip1 (34). Thus we performed IHC for p27Kip1 on C6 tumors. The lowest levels of this protein were observed in control sections (Fig. 2A, left), whereas SST-treated tumors displayed a striking increase in p27Kip1 expression levels (Fig. 2A, right). This result suggests that SST treatment may induce overexpression of p27Kip1, likely related to the reduction of ERK1/2 activation.

These data were quantified by LI of the control and treated sections for both ERK1/2 and p27Kip1 (Fig. 2B), confirming a highly significant reduction in immunopositive cells ($P < 0.01$).

**Role of individual SSTR subtypes in the antiproliferative and antiangiogenic effects of SST.** To evaluate the antiproliferative potential of individual SSTRs in vivo, first we compared the antitumoral activity of the single receptor agonists BIM-23745, BIM-23120, and BIM-23206, which were active on SSTR1, -2, and -5, respectively. These receptors were recently reported to mediate inhibition of cell growth in C6 cells in vitro (9). Mice bearing C6 gliomas were injected for 19 days with selective agonists (50 μg) or vehicle following the treatment schedule used for SST experiments. As reported in Fig. 4A, tumors developed in agonist-treated mice exhibited a reduced growth rate compared with the tumor development observed in animals in the vehicle group.

In particular, the tumor growth curve of mice treated with analog BIM-23745 (SSTR1 selective) was significantly different ($P < 0.05$) from those obtained by vehicle-treated xenografts starting from day 12, and the maximal inhibition was obtained on day 19 ($−54\%$ of the tumor volume of the control tumors, $P < 0.01$) (Fig. 4A). Tumor mass in response to SSTR2-preferential compound BIM-23120 was lower than in control mice from day 15, although it reached its maximal efficacy and statistical significance only on day 19 ($P < 0.01$). The treatment with the SSTR5-selective analog BIM-23206 significantly inhibited the growth of C6 tumors already on day 12 ($P < 0.05$) and reached the highest significance for all of the other time points up to the end of the experiment ($P < 0.01$).

Tumor mass shrinkage, in agonist-treated C6 gliomas, was paralleled by a reduction in the intratumoral microvascular densities, demonstrating a direct antiangiogenic activity that was maximal for the SSTR1 and SSTR2 agonists (Fig. 3B). BIM-23206 also showed a significant inhibition in neovessel formation, albeit to a slightly lower level (Fig. 3B).

Individual SSTR agonists induced a small increase in caspase-3 activation that was comparable with that induced by SST (data not shown).
Intracellular effectors activated by individual SSTRs. Selective SSTR agonists were also tested for their effects on Ki-67 levels, ERK1/2 activation, and the levels of p27<sup>Kip1</sup> expression, evaluated in immunostained sections, and recorded as LI at the end of the treatment (day 19). The fraction of Ki-67-positive tumor cells was significantly lowered by SSTR1, -2, and -5 agonist treatment, with BIM-23120 being the least effective. Similarly, ERK1/2 phosphorylation was greatly reduced by all the SST analogs used, confirming that the antiproliferative effects activated by these receptor subtypes are mediated by the inhibition of MAP kinase activity.

Conversely, ~5% of vehicle-treated tumor cells showed p27<sup>Kip1</sup> expression, and this value was significantly increased by all the agonists (P < 0.01; Fig. 4B). Interestingly, the effect of SST on all of these parameters was slightly more powerful than that of the individual SSTR agonists (see Fig. 2B), although the results were not statistically different.

Antiproliferative activity, antiangiogenic effects, and intracellular mechanisms involved with bispecific SSTR agonists. To obtain further insights on the possible synergism between different SSTR subtypes, we tested the antitumoral effects of the treatment with bispecific compounds BIM-23704 (SSTR1 and -2) and BIM-23190 (SSTR2 and -5) compared with those obtained by compounds effective on single receptors.

First, we compared the rate of the tumor growth obtained by the treatment with BIM-23190, BIM-23120, or BIM-23206. As shown in Fig. 5A, the mean volume of tumors of the BIM-23190-treated tumors significantly reduced the growth rate, being much more pronounced than that of BIM-23120 and almost superimposable with that of BIM-23206 with only a statistically significant difference (P < 0.05) from the control curve obtained from day 10 compared with the effect observed from day 12 using the SSTR<sub>5</sub>-selective agonist (P < 0.05).

Then, we compared the efficacy of the treatment with the individual agonists BIM-23745 (SSTR1) and BIM-23120 (SSTR2) with those of BIM-23704, which binds both receptors. The simultaneous activation of SSTR1 and SSTR2 by BIM-23704 elicited a higher biological response in terms of inhibition of tumor growth rate (statistically significant from day 12, ~75%, P < 0.01) compared with the results obtained after single receptor activation (Fig. 5B). The additivity of the effect of SSTR1 and -2 treatment was demonstrated by the growth curve for this group as being consistently below the curves for treatment with the single agonist.

As far as the modulation of tumor angiogenesis and the intracellular effectors, both of the bispecific drugs showed a significant decrease in the microvascular densities that was comparable with that induced by SST but slightly lower than that induced by the selective SSTR1 and -2 agonists (Fig. 3). Conversely, no differences were observed as far as the activation of caspase-3 (data not shown).

BIM-23704 and BIM-23190 also reduced LI for Ki-67 and phospho-ERK1/2 and upregulated p27<sup>Kip1</sup> expression with an efficacy slightly higher than that observed with the individual agonists and comparable with that obtained by SST treatment (Fig. 6).

Altogether, these results provide evidence that the effective SSTRs in C6 xenografted cells act through the same intracellular pathways, as observed for SST.

DISCUSSION

The expression and tissue distribution of SSTRs have been studied extensively, and the interest in these receptors becomes greater due to the novel therapeutic opportunities opened by the use of synthetic SST analogs for the control of hormonal symptoms and cell growth of pituitary adenomas and gastroenteropancreatic and other neuroendocrine tumors (33). A wide series of solid or hematological malignancies have been demonstrated to variably express SSTRs (54). As far as brain tumors, different SSTR subtypes have also been detected in both meningiomas (4, 10) and gliomas (17), where receptor distribution correlated with scintigraphic imaging and in vitro response to SST analogs (16). Moreover, SST and analogs showed antineoplastic activity in a variety of experimental tumor models in vivo, inhibiting the growth of transplantable...
tumors and improving the survival of tumor-bearing animals. More recently, experimental and clinical evidence proposed that SSTR agonists can be effective not only for neuroendocrine tumors (45) but also for other tumor histotypes, such as hepatocellular carcinoma (52). Nevertheless, the potential use of SSTR agonists in the control of GBM growth, although demonstrated in vitro, is not yet well characterized in vivo, specially as far as the intracellular mechanisms involved. Thus it is of particular relevance to verify the results obtained in vitro in an in vivo experimental setting. Different mechanisms have been proposed for the antiproliferative activity of SST, but a general consensus about the central role of several PTPs interfering with MAP kinase activation has now been obtained (11, 27, 41). In turn, the inhibition of ERK1/2 MAP kinase cascade (22, 53) results in an inhibition of the proteasome degradation of p27kip1 and its overexpression (22, 35, 42).

To study the in vivo efficacy of individual SSTR on glioma development and the intracellular pathways, we choose as experimental model the C6 rat glioma cell line, in which we previously characterized the PTP\(_1\)/H9257-dependent antiproliferative activity of SST (41), the SSTR profile, and the role of individual SSTRs on cell growth inhibition in vitro (9). We investigated the susceptibility of C6 tumors to the in vivo antiproliferative and antiangiogenic effects of SSTR agonist treatment after xenotransplant in nude mice. Moreover, we analyzed in immunohistochemistry studies on the C6 tumors, explanted at the end of the experimental protocols, the intracellular pathways responsible for such effects, focusing on the inhibition of ERK1/2 activation and the increase of p27kip1 expression, with both conditions being the final effects occurring after SSTR-mediated PTP\(_1\) activation (9). Since SST is known to display a very short half-life, to maintain elevated concentrations of the peptide in the tumor, we performed the treatments twice/day, using high doses (50 \(\mu\)g) of peptide. Prolonged treatment with SST significantly reduced tumor growth rate, although we did not observe a complete growth arrest. Interestingly, the efficacy of the treatment lasted for all of the length of the experiment (19 days), and no signs of receptor downregulation were observed. The antitumor effects of SST were reported to be either cytostatic (growth arrest) (26) or cytotoxic (proapoptotic) (19). The antiproliferative effects of SST did not appear to be related to the induction of cell death in C6 tumors. Measuring the activation of caspase-3, tumors and improving the survival of tumor-bearing animals.

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Thus it is of particular relevance to verify the results obtained in vitro in an in vivo experimental setting. Different mechanisms have been proposed for the antiproliferative activity of SST, but a general consensus about the central role of several PTPs interfering with MAP kinase activation has now been obtained (11, 27, 41). In turn, the inhibition of ERK1/2 MAP kinase cascade (22, 53) results in an inhibition of the proteasome degradation of p27kip1 and its overexpression (22, 35, 42).

To study the in vivo efficacy of individual SSTR on glioma development and the intracellular pathways, we choose as experimental model the C6 rat glioma cell line, in which we previously characterized the PTP\(_1\)/H9257-dependent antiproliferative activity of SST (41), the SSTR profile, and the role of individual SSTRs on cell growth inhibition in vitro (9). We investigated the susceptibility of C6 tumors to the in vivo antiproliferative and antiangiogenic effects of SSTR agonist treatment after xenotransplant in nude mice. Moreover, we analyzed in immunohistochemistry studies on the C6 tumors, explanted at the end of the experimental protocols, the intracellular pathways responsible for such effects, focusing on the inhibition of ERK1/2 activation and the increase of p27kip1 expression, with both conditions being the final effects occurring after SSTR-mediated PTP\(_1\) activation (9). Since SST is known to display a very short half-life, to maintain elevated concentrations of the peptide in the tumor, we performed the treatments twice/day, using high doses (50 \(\mu\)g) of peptide. Prolonged treatment with SST significantly reduced tumor growth rate, although we did not observe a complete growth arrest. Interestingly, the efficacy of the treatment lasted for all of the length of the experiment (19 days), and no signs of receptor downregulation were observed. The antitumor effects of SST were reported to be either cytostatic (growth arrest) (26) or cytotoxic (proapoptotic) (19). The antiproliferative effects of SST did not appear to be related to the induction of cell death in C6 tumors. Measuring the activation of caspase-3,
we suggest that this effect is mainly tumoristatic rather than apoptotic. Indeed, at least in our experimental model, although the number of apoptotic cells is slightly increased upon SST treatment, this effect involved a rather small number of cells that only marginally contributed to the antitumoral effects in C6 xenografts. Moreover, we cannot exclude also an indirect proapoptotic SST effect induced by the antiangiogenic activity of the peptide. In fact, another mechanism by which SST and its analogs may exert their antiproliferative effects is the inhibition of tumor neoangiogenesis. Growing vascular endothelial cells express several SSTR subtypes (1, 62), and SST regulation of tumor angiogenesis has been described in human glioma cells (43).

A significant reduction of the neovascularization of the xenografted tumors was observed after SST treatment, as determined by morphological examination of the hematoxylin- and eosin-stained sections and by immunohistochemical staining for the endothelial cell markers CD31 and CD34. This observation confirms that, beside a direct antiproliferative effect, an indirect antitumoral activity of SST also occurs in vivo, involving antiangiogenic mechanisms (5, 23).

To delve deeper into the mechanisms of SST-mediated tumor growth arrest in vivo, we performed immunohistochemical analysis of C6 tumors at the end of the in vivo experiments. First of all, we confirmed the reduction of the number of cells in active proliferation in SST-treated tumors as demonstrated by the significant reduction of the expression of the proliferation-associated antigen Ki-67. Then, since it was not possible analyze the specific PTP activity in the tumor sections, we decided to look at the intratumoral levels of active (phosphorylated) ERK1/2 and the expression of the CDK inhibitor p27kip1, two intracellular mediators of cell proliferation whose modulation is an indirect index of PTP activity. We show for the first time that SST in vivo antitumor effects are mediated by the inhibition of ERK1/2 activation and upregulation of p27kip1. Much evidence suggests that deregulation of p27kip1 plays a pivotal role in the progression and tumor behavior of many malignant diseases due to its well-established inhibitory effects on cell proliferation, thus representing an interesting pharmacological target, especially in those cancers refractory to the conventional therapies, like human GBM (47). In GBM, cell lines and its overexpression blocked cancer cell growth, local invasion, and tumor-induced neoangiogenesis (56), whereas in C6 cells, increased p27kip1 stability and translational activation lead to cell growth arrest and differentiation (44).

Our data reveal for the first time that the modulation of the activity of these cell cycle regulators is responsible for SST cytostatic effects in vivo. Interestingly, the identification that the same pathways are responsible for the SST activity both in vitro (9) and in vivo (this study) strongly validates the role of the regulation of ERK1/2 and p27kip1 in SST effects.

The second relevant information coming out of our in vivo study is the identification of the role of individual SSTRs in the antiproliferative effects of SST. In fact, besides SSTR2, which was identified to be highly expressed in most neuroendocrine neoplasias, other SSTR subtypes are often expressed (and coexpressed) in several human tumors. For instance, the efficacy of clinically approved SST analogs on tumor growth is attributed to SSTR2 and SSTR5, whose expression predominates in growth hormone-secreting adenomas (36). Moreover, the observation that SSTR subtypes on the same cancer cell can form homo- and heterodimers with different functional characteristics, as reported for SSTR2, which heterodimerizes with SSTR5 (55), may support the development of therapeutic approaches that take into account possible synergistic mechanisms.

To evaluate the specific role of single SSTRs in the antiproliferative effects of SST and the intracellular pathway involved, we treated C6-bearing mice with selective agonists for SSTR1, -2, and -5. Although SSTR3 is also expressed in C6 cells, we did not evaluate its effects on tumor growth since no peptide agonists were available, and its activation does not affect C6 cell proliferation, in vitro (9). We demonstrate that SSTR1, -2, and -5, when activated by preferential agonists, induced inhibition of tumor growth. However, a different effectiveness was observed, with the SSTR5-specific compound being the more powerful and the SSTR2 agonist the weakest one. Interestingly, the same ranking of potency for these compounds was also observed in vitro using the same cells (9), further validating this observation.

These data support the hypothesis that SST-mediated control of tumor growth in vivo may occur mainly via a direct effect and a possible concomitant activation of multiple receptors expressed by C6 cells. The finding that C6 tumor proliferation and neoangiogenesis was inhibited by SST might indicate the likelihood that SSTR1, -2, and -5 are functional in the proliferation of tumor endothelial cells. However, from the present study we cannot discriminate whether the activation of SSTR1, -2, and -5 occurs also at endothelial cell level or the inhibition of neoangiogenesis is caused, indirectly, by the inhibition of the production of proangiogenic factors by the C6. In fact, both mechanisms were previously reported to be induced after SST treatment (2, 43). Therefore, a further characterization of the mechanism of SSTRs in the inhibition of glioma angiogenesis will be required.

Since the antiproliferative response induced by SST may be due to the cumulative activation of different SSTRs expressed by C6 glioma xenografts, we compared the tumor growth curves obtained from BIM-23745, BIM-23120, and BIM-23190 and p27kip1 in saline, SST, and bispecific compounds treated tumors (SSTR2 and -5 agonist BIM-23745, BIM-23120, and BIM-23190, and SSTR1 and -2 agonist BIM-23704), reported as average from 5 individual tumors for each experimental treatment.

Fig. 6. Immunohistochemical evaluation of the intracellular mechanisms involved in the SSTR agonist antiproliferative activity in C6 experimental tumors. Labeling index (% ± SE) of immunopositive cells for Ki-67, p-ERK1/2, and p27kip1 in saline, SST, and bispecific compounds treated tumors (SSTR2 and -5 agonist BIM-23745, BIM-23120, and BIM-23190, and SSTR1 and -2 agonist BIM-23704), reported as average from 5 individual tumors for each experimental treatment.
23206 treatment with those derived from the tumor exposure to SSTR1 and -2 and SSTR2 and -5 agonists (BIM-23704 or BIM-23190), with SSTR1 and SSTR2 being the SSTRs most frequently detected in brain tumors and SSTR2 and SSTR5 the targets of clinically used SST agonists octreotide and lanreotide.

The simultaneous stimulation of SSTR1 and SSTR2 by BIM-23704 resulted in an increased tumor growth inhibition compared with individual receptor analogs, whereas the response to the concomitant activation of SSTR2 and SSTR5 was comparable with that obtained after the SSTR5 receptor activation.

However, although the maximal inhibition of tumor growth occurred via SSTR5 activation, SSTR1 and -2 agonists showed a more powerful inhibition of microvascular density formation. Thus the combined activation of multiple SSTRs (as occurs with the natural peptide) may induce a more complete tumor growth not only for the synergism in the direct antiproliferative activity of the different receptor subtypes (see the effects obtained using bispecific agonists) but also for a more complete inhibition of neovascularization. Moreover, the different efficacy of SSTR agonists on direct tumor growth inhibition and neovessel formation clearly indicates that the two activities are somehow independent from each other and that the lower vascularization in the treated tumors does not reflect a simple consequence of a smaller tumor volume.

In addition, we demonstrated that the antitumor activity of each single receptor agonist is mediated by a common intracellular pathway (inactivation of ERK1/2 and upregulation of p27^Kip1), as also observed in vitro (9). This effect, although quite heterogeneous within tumors, was evident after the first days of treatment showing that activation of SSTRs resulted in a reduced number of cells in which ERK1/2 was active and thus less cells that entered in the proliferative phases of the cell cycle. Interestingly, although significant differences were observed on tumor development, the effect of the agonist on phospho-ERK1/2 and p27^Kip1 LI was comparable for all of the treatments. A possible explanation for this apparent discrepancy may reside in the fact that the immunohistochemical analysis was performed at the end of the tumor growth experiments when all of the treatments induced their maximal effect, making it more difficult to identify significant differences in the regulation of the intracellular signaling. In any case, in the treatments involving multiple receptor activation (SST, BIM-23704, and BIM-23190), a slight, albeit not significant, increase in the ERK1/2 inhibition and p27^Kip1 overexpression was observed.

That SSTR1-, -2-, and -5-mediated effects were mainly cytostatic in nature was confirmed by the significant increase in the expression of the CDK inhibitor p27^Kip1 altogether with a significant reduction of ERK1/2 activation. Similar results were obtained using SSTR2 and -5 selective analogs in other cell types (29, 35).

Our results underline a possible novel interesting aspect of the antiproliferative activity displayed by SST and its analogs in experimental tumors. In fact, it was demonstrated recently that the in vivo tumorigenicity of C6 cells is dependent on the presence of a consistent subpopulation of CSCs (58, 65). The retention of “stemness” properties by C6 cell subpopulations provides a novel relevance to this model since, as demonstrated in human glioblastomas, CSCs represent the real target for anticancer drugs (32). However, these cells develop different mechanisms of resistance to many traditional anticancer compounds responsible for the therapeutic failure. Since CSCs provide the cellular basis of the initiation and recurrence of gliomas, the elimination of CSCs within the tumor mass represents the only effective therapeutic strategy for refractory brain tumors. In this view, the present study may support the hypothesis that SST being able to affect the growth of C6 gliomas in vivo likely interferes with the persistence of CSCs within the tumors to cause a significant suppression of their growth. Although in this study we did not directly address this issue, this observation may represent the starting point for a novel reevaluation of the potential antitumoral activity mediated by SST agonists through the inhibition of CSC survival.

In conclusion, we report that 1) SSTR1, -2, and -5 activation leads to cell growth inhibition of C6 experimental gliomas in vivo, acting on both tumor cell proliferation and neoangiogenesis; 2) the effects mediated by the different SSTRs were mainly cytostatic, with very limited induction of apoptosis observed; 3) SSTR5 activation showed the highest efficacy, although the combined activity of different SSTR subtypes resulted in an additive effect; and 4) the inhibition of ERK1/2 and the upregulation of p27^Kip1 were the common intracellular mechanisms mediating the antitumoral effects induced by all the SSTR subtypes.

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