SMS 201.995 Inhibits in Vitro and in Vivo Growth of Human Colon Cancer

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ABSTRACT

The effect of a long-acting somatostatin analogue SMS 201.995 (SMS; Sandoz) on basal and gastrin-stimulated growth of 4 human colon cancer lines was studied in vitro and in vivo. Proliferation assay was done with overnight [35S]selenomethionine uptake after 5 days of incubation. Gastrin concentrations used were 5e-10 M and 1e-7 M. SMS concentrations used were 5e-10 M and 1e-7 M. SMS concentrations used were 5e-10 M and 1e-7 M. LIM 2412 was significantly stimulated. Based on in vitro growth characteristics, LIM 2412 and LIM 2405 were selected for xenograft study. The dose of 50 µg/kg/day was arrived at after a preliminary study showed it to be safe and effective. The LIM 2412 xenografts in the SMS-treated animals were 473.3 ± 99.9 (SD) versus 838.1 ± 111.3 mm³ in control (P < 0.05) after 20 days. The LIM 2405 tumors were also significantly inhibited (81.2 ± 30.0 versus 245.7 ± 48.3 mm³, P < 0.01). The effect of SMS appeared to be reversible. Oral SMS at 200 µg/kg/day was not absorbed. This study suggests that SMS may have direct antitumor effects in human colon cancer.

INTRODUCTION

Colon cancer is one of the most common causes of cancer deaths in the Western World. Few patients present with early disease and the treatment of advanced colorectal cancer is far from satisfactory. Of all patients with colorectal cancer, over two-thirds still die of their disease. Clearly, optimal therapy remains the surgical excision of early disease. The contributions of cytotoxic chemotherapy and radiotherapy have been modest and, while there is recent evidence of real value for both modalities in adjuvant settings (1, 2), their role in advanced cases remains the surgical excision of early disease. The contributions of an impetus to seek an alternative therapy that offers better results. One of the areas under investigation is the role of hormones.

The role of sex steroids in breast and prostatic cancers is well established, and hormonal manipulation is an important therapeutic modality. Several peptide hormones, such as gastrin and cholecystokinin, have been shown to exert trophic effects on the growth and differentiation of normal, as well as malignant, gastrointestinal cells in vitro and in vivo (4-7). The mechanism of action is probably mediated by specific hormone receptors (8-10); thus, there were efforts to develop receptor antagonists as anticancer agents (11-14). One of the most important naturally occurring antitrophic hormones is somatostatin (15). It was shown to inhibit cellular proliferation in vitro (20, 21). Its half-life in plasma was 113 min after a single release (24). Its half-life is approximately 113 min after a single s.c. injection of 50 or 100 µg in humans (25). It has been widely used in humans with little toxicity (26). It is now an accepted treatment for, among others, the neuroendocrine tumors of the gut, such as carcinoids, insulinomas, and vipomas (27-30). Whereas its effect may be mostly symptomatic, there are also reports that it actually resulted in tumor regression (31, 32). It has also been shown to have antiproliferative effects against cultured human breast cancer cells and xenografts (33, 34).

Whether SMS 201.995 is effective against nonendocrine gastrointestinal tumors is still unclear (35). It inhibited the proliferation of a human gastric cancer cell line in vitro and in vivo (36) as well as rat and human pancreatic cancer cells in vitro and as xenografts (37-41). It also reportedly reduced pentagastrin-mediated trophic effects on MC-26 mouse colon cancer (42), although other studies found no effect on rat colorectal carcinogenesis (43). The response of human colon cancer to SMS 201.995 has not been reported previously. In this study, we have investigated the possibility that SMS 201.995 may be able to inhibit the growth of established colon cancer in the same way that tamoxifen can slow the growth of breast cancer. The effect of SMS 201.995 on the growth of some human colon cancer cell lines was studied in vitro and as xenografts in nude mice.

MATERIALS AND METHODS

In Vitro Experiment

Cell Lines. Four human colorectal cancer lines were used. They were established at the Ludwig Institute in Melbourne and were kindly provided by Dr. R. H. Whitehead.

1. LIM 1215 was derived from a colonic adenocarcinoma in an orthotopic liver metastasis. The biopsy showed poorly differentiated adenocarcinoma. The cultured cells were small and pleomorphic, with a doubling time of 36 h.

2. LIM 2412 derived from a poorly differentiated adenocarcinoma of the ileocaecal valve, which was described as a poorly differentiated ulcerated carcinoma that extended through the full thickness of the muscle wall. In vitro, it grows as organoids consisting of columnar and goblet cells, which are morphologically and functionally organized, with cells arranged around a central lumen (45).

3. LIM 2405 derived from a poorly differentiated colonic adenocarcinoma. The cultured cells grow as adherent monolayers, with an estimated doubling time of 30-36 h.

4. LIM 2412 also derived from a poorly differentiated colonic adenocarcinoma. In vitro, it grows as floating organoids that are morphologically and functionally organized, with a doubling time of approximately 30-36 h.

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The abbreviations used are: LIM, Ludwig Institute, Melbourne; SMS, SMS 201.995; FCS, fetal calf serum. A nonstandard expression for concentrations is used throughout the article. To translate into the standard form, follow this example: 2e-12 M = 2 × 10^-12 M.

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Maintenance of Cells. The cells were maintained in RPMI with 5% FCS, supplemented with insulin 100 µg/ml, l-glutamine 200 µM, sodium pyruvate 100 µM, penicillin 5000 I.U./ml, and streptomycin 5000 µg/ml (Cytosystems Pty. Ltd., New South Wales). They were grown in 80-cm² tissue culture flasks and kept in a humidified atmosphere of 5% CO2/95% air at 37°C.

Synchronization. The cells were synchronized by growing in serum-free media for 48 h, followed by washing and changing back into RPMI with 5% FCS plus thymidine (46). The concentration of thymidine (Sigma Chemical Co., St. Louis, MO) used was 2 µM after it was shown to be optimal for these cell lines.

Proliferation Study. Proliferation assays with LIM 1863 and LIM 2412 were done using whole organoids, since initial trials with single cell suspensions by disrupting the organoids resulted in a high percentage of nonviable cells. Single cell suspensions of LIM 1215 and LIM 2405 were used.

Using an Eppendorf Multipette, the cells were then plated in 96-well plates at 30 organoids/well (approximately 5 x 10⁴ cells) for LIM 1863 and LIM 2412 and 2 x 10⁴ cells for LIM 1215 and LIM 2405, each well containing 100 µl. Gastrin (Sigma) was added at concentrations of 5e-10 M and 1e-7 M. The concentration of SMS was 2e-12 M to 2e-7 M. Each combination of gastrin and SMS, together with separate wells serving as control, was done in triplicate and the experiments were done twice. The cells were re-fed on the 3rd and finally on the 5th day with the corresponding concentration of gastrin and SMS in RPMI with 5% FCS. [Sej3]Selenomethionine (0.1 µCi/well; Medegenix Diagnostics, Belgium) was pulsed on the 5th day and the plates counted the following day with the 1272 Clini gamma (LKB-Wallac, Finland), using the technique we described earlier (47, 48). On separate wells with the same number of cells exposed to SMS at 2e-10 M and 2e-7 M and gastrin at 5e-10 M and 1e-7 M, the cell count was done with a hemocytometer after 5 days to confirm that [Sej3]Selenomethionine uptake corresponded to cell number.

The triplicates were averaged and the results expressed as a percentage of control. Student's t test for independent samples was performed.

Xenograft Experiment

Mice. The male, athymic, nude BALB/c mice were purchased from the Animal Resource Center, Western Australia. They were flown to Sydney and kept in a laminar-flow filtered air room with 12-h light and dark cycle, and fed irradiated rodent chow (Barastoc Stock Feeds Pty. Ltd., Victoria, Australia) and autoclaved acidified water ad libitum. They weighed between 19 and 21 g at the start of the experiment.

Preparation of Xenografts. The cells from the 4 cell lines were injected s.c. into 2 mice each: LIM 1215 and LIM 2405 were given at 2 x 10⁶ cells in 150 µl, and LIM 1863 and LIM 2412 were at 100 organoids in 150 µl. Tumor nodules were palpable after a week in the LIM 2412 and after 4 weeks in the LIM 2405 lines. They were thus selected for the in vivo experiment. Cell lines LIM 1215 and LIM 1863 failed to grow as xenografts. Repeated attempts in another 20 nude mice were not fruitful either, although successful implants were reported previously (44, 45).

Tumor fragments were obtained from serially transplanted LIM 2405 and LIM 2412 xenografts. The mice were killed when the tumors were approximately 1 cm in largest dimension. The tumors were excised and minced into 1-mm³ pieces. After washing with ice-cold RPMI with 5% FCS, the fragments were ready for implantation. Tumor implants were done s.c. in identical areas in nude mice to obviate the effect of regional differences in growth rate as a variable (49). Saline, SMS, or gastrin was delivered via Alzet pumps (model 2002; Alza Corp., Palo Alto, CA), which were implanted s.c. The tips of the pumps were directed away from the tumor site. The operative procedures were done aseptically with the mice under Hypnorm (fentanyl/fluanisone; Janssen, Belgium) neuroleptic analgesia at 30 mg/kg i.p.

Tumor size was measured twice weekly using a Vernier caliper, and the largest perpendicular dimensions were obtained, with tumor volume computed as 1/2 xy². Measurement of body weight was done weekly. The pumps were excised after 20 days as per manufacturer's instruction, to prevent leakage of hypertonic solution. The mice were killed after the experiment was over. The lungs, liver, and abdominal cavity were examined for tumors. All experimental procedures were approved by the Institutional Animal Care and Ethics Committee.

Five experiments were done:
1. In the preliminary experiment, because the correct dose of SMS for colorectal cancer is unknown, we decided to test 3 different doses in our mice to determine its safety as well as effectiveness in inhibiting tumor growth. Twenty nude mice bearing LIM 2405 tumors were randomized into 4 equal groups, each mouse receiving one Alzet pump that contained saline in the control group, and SMS at 5, 25, and 50 µg/kg/day, respectively, in the 3 experimental groups. The pumps were put in 7 days after tumor transplantation, and the experiment ended after 19 days.

2. Twenty mice were allocated for LIM 2412 and another 20 for LIM 2405 xenografts. For each type of xenograft, the mice were divided randomly into 4 groups, to receive 2 Alzet pumps containing: (a) saline in both pumps, which serves as control; (b) gastrin and saline; (c) SMS and saline; and (d) gastrin and SMS. The dose of gastrin was 5 µg/kg/day and SMS at 50 µg/kg/day. The pumps were implanted 1 day after tumor transplantation in the LIM 2412 line and 8 days after in the LIM 2405 line. The experiments were terminated after 19 and 21 days, respectively.

3. Twenty mice bearing LIM 2405 tumors were randomized into 2 groups that received saline and SMS 50 µg/kg/day, respectively. The pumps were implanted 8 days after the tumors. Mice in the control group were killed when 4 started to have skin ulceration on the 21st day. The pumps in the SMS group were excised and new pumps inserted after the same dose. The mice were initially implanted. The experiment was terminated on the 35th day, when 3 mice in the SMS group started to have skin ulceration.

4. Ten nude mice were implanted with LIM 2412 xenografts and randomized into 2 groups that received saline and SMS 50 µg/kg/day, respectively. Treatment was started on the same day as tumor implantation and continued for 14 days. Tumour size was measured from day 5 to 14. At day 15, the pumps of all 10 mice were excised. Tumor measurement resumed on day 19 and continued up to day 29.

5. Ten mice bearing LIM 2412 tumors were randomized into 2 groups. The control and experimental groups had the same condition, except that the latter had SMS 200 µg/kg/day added to the drinking water. Treatment started the day after tumors were implanted. The experiment was terminated after 21 days. The decision on p.o. dosage of SMS was largely empirical. Oral absorption was not very good, as reported earlier (50). The dose of 200 µg/kg/day p.o. was 4 times the s.c. infusion dose of 50 µg/kg/day.

SMS Radioimmunoassay

Sera were obtained from mice in these experiments for radioimmunoassay for SMS. The radioimmunoassay kit was kindly furnished by Dr. Peter Marbach of Sandoz, Switzerland. Briefly, sera were incubated with known amounts of 125I-SMS tracer and anti-SMS sera for 24 h at 4°C. Then charcoal was added and the solution incubated for another 10 min at 4°C, followed by centrifugation at 3500 rpm for 5 min. Associated radioactivity in the supernatant was counted and serum SMS concentration was derived with the 1272 Clini gamma (LKB-Wallac, Finland).

RESULTS

In Vitro. The organoids remained nonadherent and not confluent, and appeared bigger and more numerous after 5 days. The adherent cell lines were confluent by the 5th day. Three of the 4 cell lines were inhibited by SMS in vitro, and one was not.

Direct count of cell number showed that they corresponded to 79Se-selenomethionine uptake when expressed as percent of radioactivity in the supernatant compared to the control (Table 1). Significant stimulation occurred with LIM 2405 and LIM 2412 at 1e-7 M gastrin, whereas SMS inhibited LIM 1215 at 2e-10 M and LIM 2405 and LIM 2412 at 2e-7 M.
concentration ($P < 0.05$). However, the percentage change observed with direct cell count appeared smaller than that of $[^7]	ext{S}$Seleomenithionine uptake, e.g., at $1 \times 10^{-7}$ M gastrin, LIM 2412 cells were 23 and 41% of control, respectively.

For LIM 1215, the addition of gastrin failed to stimulate cell proliferation. There was a progressive but modest trend of proliferation. There was a progressive inhibition with higher concentration decreased by as much as 22.5% as compared to control, $P < 0.05$. At basal condition, $5 \times 10^{-10}$ M gastrin slightly stimulated cell proliferation by 8.5% ($P > 0.05$), but even higher concentration $1 \times 10^{-7}$ M inhibited it by 11.1% ($P > 0.05$).

In Vivo. All 1-mm$^3$ pieces of implanted tumors grew successfully as xenografts. They were measurable by 5–7 days when edema subsided. The LIM 2415 was multilobulated, while the LIM 2405 appeared smooth and, although initially s.c. and mobile on the posterior chest wall, both invaded the skin and became fixed to it. There was no macroscopic evidence of peritoneal, liver, or pulmonary metastases at sacrifice.

The mean body weights of the groups were not significantly different from each other, increasing by an average of 10% every week. There were no adverse effects seen in the animals that received gastrin or SMS.

In the experiment with different doses of SMS, the mean tumor sizes in the $50 \mu g$/kg/day SMS-treated animals were approximately half of the control group ($200.2 \pm 53.9$ (SD) versus $405.8 \pm 64.9$ mm$^3$, $P < 0.05$) by 13 days. Those in the $25 \mu g$/kg/day group were also inhibited, but not significantly ($220.3 \pm 86.4$ mm$^3$, $P > 0.1$). After 19 days, however, the tumors in the $25 \mu g$/kg/day group were already significantly inhibited ($467.3 \pm 214.7$ versus $1437.5 \pm 212.5$ mm$^3$, $P < 0.02$). The tumors in the $5 \mu g$/kg/day SMS-treated animals were not significantly inhibited throughout the experiment (Fig. 5).

In the second experiment, with gastrin infused at $5 \mu g$/kg/day and SMS at $50 \mu g$/kg/day, the LIM 2412 tumor growth was significantly inhibited by SMS over the observation period (Fig. 6). By the 19th day, the tumor volume in SMS-treated animals was only approximately half that of the control group ($473.3 \pm 99.9$ versus $838.1 \pm 111.3$ mm$^3$, $P < 0.05$). While gastrin infusion did not stimulate tumor growth, it appeared to have reversed the inhibitory effect of SMS in the group that received both compounds. As for the LIM 2405 xenografts, by the end of the treatment period, the tumors in the SMS-treated groups were also significantly smaller than those that did not receive the drug ($81.2 \pm 30.0$ versus $245.7 \pm 48.3$ mm$^3$, $P < 0.01$). Although the group that received both gastrin and SMS appeared to have the greatest inhibition, it was not significantly different from the group that received SMS only (Fig. 7).

In the third experiment, the mean tumor sizes in the SMS-treated animals were only one-fifth that of the control group ($473.3 \pm 99.9$ versus $838.1 \pm 111.3$ mm$^3$, $P < 0.05$). While gastrin infusion did not stimulate tumor growth, it appeared to have reversed the inhibitory effect of SMS in the group that received both compounds. As for the LIM 2405 xenografts, by the end of the treatment period, the tumors in the SMS-treated groups were also significantly smaller than those that did not receive the drug ($81.2 \pm 30.0$ versus $245.7 \pm 48.3$ mm$^3$, $P < 0.01$). Although the group that received both gastrin and SMS appeared to have the greatest inhibition, it was not significantly different from the group that received SMS only (Fig. 7).

In the fourth experiment, tumor growth was slowed by SMS during the first 14 days when treatment occurred, with the tumor doubling time greater than control: 5.7 versus 4.3 days. However, when treatment ceased, the doubling times were reversed: 5.0 versus 7.0 days, with the tumors previously treated.
SMS INHIBITS COLON CANCER

Fig. 2. Effect of SMS on LIM 2405 in vitro. Progressive dose-dependent inhibition by SMS, with significant inhibition at 2e-8 M and 2e-7 M (P < 0.05).

Fig. 3. Effect of SMS on LIM 2412 in vitro. Significant inhibition by SMS occurred with basal and gastrin-stimulated groups. Cells under le-7 M of gastrin had the most significant inhibition when SMS concentration was 2e-10 M or more (/> 0.01).

Fig. 4. Effect of SMS on LIM 1863 in vitro. There was no significant SMS effect at doses less than 2e-8 M. At that dose, there was a significant increase in proliferation (P < 0.005).

Fig. 5. Effect of different dosages of SMS on LIM 2405 xenografts in nude mice. Treatment started on day 7. Mean tumor size in mice treated with both 25- and 50-μg/kg/day doses of SMS was inhibited after 19 days, with the latter dose showing a significant effect earlier also at 13 days (P < 0.05). The 5-μg/kg/day dose was ineffective.

Fig. 6. Effect of SMS on LIM 2412 in nude mice. Treatment started on day 1 and continued for 14 days. There were 5 mice in each group. The average tumor size in the SMS-treated group was significantly smaller than the control group at the 19th day (P < 0.05).

DISCUSSION

This is the first study that demonstrated that SMS 201.995 inhibited the growth of human colon cancer in vitro and in vivo. Moreover, the xenograft study, which showed that mean body weights were not different among groups, further suggested that SMS may have a direct anti-tumor effect and that it is unlikely to be a nonspecific effect. The growth inhibition observed in vivo was around 50% or more compared to control after 2-3 weeks of treatment, and it took twice as long for the SMS-treated tumors to achieve the size of untreated control. While no effect was seen with an p.o. dose 4 times that of the parenteral dose, this was because absorption of SMS in the gastrointestinal tract was poor. A much higher dose would be necessary to achieve the same serum level (50).

However, unlike the known effects of cytotoxic chemotherapy, there was no decrease in tumor size. Although there were isolated reports of actual shrinkage of metastatic nodules in endocrine tumors (31, 32), the most likely effect may be a...
control after 20 days (P < 0.05), but were not statistically different from each other. Average tumor size in the gastrin group was also not significantly different from the control group.

There were 5 mice in each group. The average tumor size in the gastrin group was also not significantly different from the control group. It took twice as long to achieve the mean tumor size in the treated group as it did to achieve the same tumor size in the control group.

Fig. 7. Effect of SMS on LIM 24-5 in nude mice. Treatment started on day 8 and continued for 14 days. There were 5 mice in each group. The average tumor sizes in the 2 SMS-treated groups were significantly smaller than that of the control after 20 days (P < 0.05), but were not statistically different from each other. Average tumor size in the gastrin group was also not significantly different from the control group.

Fig. 8. Effect of continuous SMS treatment on LIM 2405 xenografts in nude mice. It took twice as long to achieve the mean tumor size in the treated group as it did to achieve the same tumor size in the control group.

decreased growth rate, especially for colorectal cancer. This is reinforced by the fact that the effect of SMS on cancer growth was found to be transient, and persisted only with continuous treatment. Nevertheless, we believe that if a nontoxic and safe drug is found that can slow the growth of colon cancer, it represents an advance in the management of a disease that has so far proved difficult to treat effectively.

The proper dose of SMS for cancer treatment still needs to be resolved. We have found that 25 and 50 μg/kg/day appeared to be safe and effective, with the latter dose shown to be effective after a shorter duration of treatment. When given as continuous s.c. infusion, the 50-μg/kg/day dose gave rise to a serum SMS level of 1223.9 ng/ml, which is equivalent to 1.2 x 10^-6 M. The low dose of 5 μg/kg/day translated to 1.4 x 10^-7 M in the serum. Thus, for tumor inhibition to occur, the effective serum concentrations of SMS were higher than that observed in in vitro studies. However, the tissue SMS level may not be the same as the serum level. The question of bioavailability of SMS in cancer tissue will have to be addressed.

The exact mechanism of action of somatostatin is not clear. Certainly, the presence of receptors may be an important factor, and there is some evidence that somatostatin acts through receptors in inhibiting the growth of some neuroendocrine tumors (51-53). There are, however, other mechanisms that are likely to be involved also, such as: (a) inhibition of the secretion of growth hormone, insulin, and gastrointestinal hormones; and (b) direct or indirect inhibition of somatomedin C or other growth factors (54).

From other studies, it was suggested that somatostatin can inhibit gastrin synthesis, release, or action (55, 56), thereby partly explaining its anti-cancer effects. This is a particularly attractive hypothesis because gastrin is an important trophic hormone for gastrointestinal mucosal cells, and we have previously established that patients with colorectal cancer have higher serum gastrin concentrations (57). The recent suggestion by our group and others that some gastric and colorectal cancers synthesize a gastrin-like peptide may also be significant (48, 58), since SMS may act directly or indirectly on that peptide. However, in our study, the exact relationship between somatostatin and gastrin was not clear. In vitro studies with LIM 2412 showed that a high concentration of gastrin made the cells more susceptible to somatostatin inhibition, thus suggesting a role for gastrin or gastrin receptor in the inhibitory mechanism by SMS. The same pattern was not seen with the in vitro results of other cell lines, however. In contrast to the in vitro data, the xenograft experiment with LIM 2412 showed that SMS strongly inhibited tumor growth in mice and that the s.c. infusion of gastrin by itself did not enhance growth, nor did it potentiate somatostatin inhibition when they were given together. Instead, it appeared to have reversed the inhibitory
effect of SMS. Gastrin did not stimulate in vivo growth of LIM 2405, nor did it affect the inhibitory action of SMS. Thus, it is unlikely that SMS is acting solely through gastrin or gastrin receptors in the experiments we observed.

At present, we are still unable to predict which tumors are likely to respond to somatostatin treatment. We should also remember that the observations in this study may well be a clonal phenomenon in that, while many cell lines may be inhibited by SMS, primary cancer without such a regulatory system may not. While we are certainly still a long way from knowing that SMS 201.995 will be a useful treatment for patients with colorectal cancer, we regard our results as most encouraging. Additional studies will be needed to relate somatostatin receptor status to response, accurately define dosage requirement, and study the effect on tumors in a more physiological setting as well as investigate any effect on the metastatic process.

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